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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gary Ruvkun et al.	Art Unit:	1636
Serial No.:	09/963,693	Examiner:	S. Kaushal
Filed:	September 25, 2001	Customer No.:	21559
Title:	THERAPEUTIC AND DIAGNOSTIC TOOLS FOR IMPAIRED GLUCOSE TOLERANCE CONDITIONS		

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APPEAL BRIEF ON APPEAL PURSUANT TO 37 C.F.R. § 41.37

In support of Appellants' Notice of Appeal that was filed in connection with the above-captioned case on October 21, 2004, and with reference to the final Office Action that was mailed in this case on April 19, 2004, submitted herewith is Appellants' Appeal Brief.

TABLE OF CONTENTS

Real Party in Interest

The real party in interest in this case is The General Hospital Corporation, to whom all interest in the present application has been assigned.

Related Appeals and Interferences

There are no pending appeals or interferences related to this case.

Status of Claims

Claims 1 and 3 are pending and claims 2 and 4-6 have been canceled. Claims 1 and 3 were finally rejected in a final Office Action mailed on April 19, 2004, and are appealed.

Status of Amendments

In the Advisory Action mailed on November 26, 2004, the Office indicated that the amendments set forth in Appellants' October 19, 2004 reply will be entered for the purposes of Appeal. These amendments are reflected in the appended claims.

Summary of Claimed Subject Matter

Appellants' invention generally features a method of diagnosing an impaired glucose tolerance condition, obesity, or a propensity thereto in a patient. This method

involves analyzing the level of PTEN expression or activity in a sample isolated from the patient, where an increase in the level of PTEN expression or activity relative to a control sample is an indication of an impaired glucose tolerance condition, obesity, or a propensity thereto (claim 1). In a desirable embodiment, the level of PTEN expression or activity is analyzed by measuring PTEN lipid phosphatase activity (claim 3).

The claimed methods are described in Appellants' specification. For instance, at page 117, lines 3-7, the specification teaches that a reduction in PTEN activity potentiates insulin and/or insulin-like growth factor signaling, whereas an increase in PTEN activity causes insulin resistance downstream of the insulin receptor. Thus, the specification establishes a link between PTEN levels and an impaired glucose tolerance condition or obesity (see also, e.g., Figures 38A to 38F). Further, at page 119, lines 2-5, the specification teaches methods for analyzing the level of PTEN activity, for instance, by using a lipid phosphatase assay. Alternatively, at pages 150-152, the specification teaches methods for analyzing PTEN expression levels.

Grounds of Rejection to be Reviewed on Appeal

Appellants submit that the Office erred, in its April 19, 2004 Office Action, in finally rejecting claims 1 and 3 under 35 U.S.C. § 112, first paragraph, for an asserted lack of enablement.

Argument

The present enablement rejection of claims 1 and 3 is unsupported by the evidence provided by the Office, and, in view of the teachings of Appellants' specification, is in error and should be reversed.

I. The References Cited by the Office Do Not Support a Lack of Enablement

In the final Office Action mailed on April 19, 2004, the Office cited several references in support of the assertion that "development of impaired glucose tolerance and obesity is multi-factorial and complex," and used this assertion as a basis for the enablement rejection. In particular, the Office has cited Hirosumi et al. (*Nature* 420:333-336, 2002; hereafter "Hirosumi"), Shulman (*Clin. Invest.* 106:171-176, 2000; hereafter "Shulman"), Lönnqvist et al. (*Nat. Med.* 1:950-953, 1995; hereafter "Lönnqvist"), Fontaine (*JAMA* 289:187-193, 2003; hereafter "Fontaine"), Kahn and Flier (*J. Clin. Invest.* 106:473-481, 2000; hereafter "Kahn"), and Ogg and Ruvkun (Mol. Cell 2:887-893, 1998; hereafter "Ogg"). In view of these references, the Office has taken the position that (Advisory Action):

> [O]besity is not only the result of genetic variations but is also the outcome of personal behavioral [*sic*] and life style. Regarding impaired glucose tolerance the earlier office action clearly provides the evidence, which establishes the unpredictability in the art. (citation omitted)

Based on this, the Office asserts that Appellants' claimed method for diagnosing impaired

glucose tolerance conditions or obesity by analyzing the level of PTEN in a sample lacks enablement. Appellants respectfully disagree.

It is Appellants' position that none of the cited references calls into question the workability of the claimed PTEN-based diagnostic assays for impaired glucose tolerance conditions or obesity. Looking at each reference in turn, Hirosumi merely describes a role for c-Jun amino-terminal kinases in obesity and insulin resistance. Shulman provides an overview of the cellular mechanisms that contribute to insulin resistance. Lönnqvist deals with understanding obesity and developing routes for its pharmacological treatment; in particular, Lönnqvist describes the overexpression of the obese (ob) gene in adipose tissue of obese human subjects. And Fontaine describes differences in life expectancy observed for obese versus normal human subjects. Hirosumi, Shulman, Lönnqvist, and Fontaine uniformly fail to address the role of PTEN in mammalian glucose metabolism and fail to address the possible use of PTEN level or activity in diagnosing an impaired glucose tolerance condition or obesity.

The fifth cited reference, Kahn, provides a review of the role of insulin resistance in diabetes and the relationship of obesity to insulin resistance and type 2 diabetes. In the April 19, 2004 Office Action, the Office, citing Kahn, asserts (page 9):

> The applicant fails to consider the complexities involved in the mammalian insulin signal transduction pathway especially in context with impaired glucose tolerance and the development [of] obesity.

Appellants again disagree. Appellants fully appreciate the complexities of impaired glucose tolerance conditions and obesity, but point out that enablement of the claimed methods does not require either a complete understanding of the insulin signaling pathway or all mechanisms underlying impaired glucose tolerance or obesity. Rather, enablement of the present claims requires only that the level of PTEN expression or activity, relative to a control, be indicative of an impaired glucose tolerance condition or obesity, a correlation that has support in Appellants' specification and, as discussed below, in the publications of others following Appellants' discovery. Appellants further note that a great many medical conditions are complex, and yet tests for their diagnosis nonetheless exist. This basis alone cannot call into question the enablement of the claimed methods.

Further, regarding Kahn, Appellants point out that this reference actually supports the role of PTEN in insulin signaling. In particular, Kahn teaches (page 473, left column):

> Insulin's metabolic effects are mediated by a broad array of tissue-specific actions that involve rapid changes in protein phosphorylation and function, as well as changes in gene expression. The fundamental biologic importance of these actions of insulin is evidenced by the fact that the insulin signaling cascade which initiates these events is largely conserved in evolution from *C. elegans* to humans. (emphasis added)

Indeed, it was Appellants' work in *C. elegans* that identified the role of DAF genes (such

as *daf-18*) in impaired glucose tolerance conditions and obesity, and that led to the discovery that *daf-18*'s mammalian counterpart, PTEN, plays a central role in insulin signaling and can therefore be used as a diagnostic marker for impaired glucose tolerance conditions. The Office, as a basis for its enablement rejection, has questioned this correlation between the pathways in *C. elegans* and mammals, and cited Kahn as supportive of its position. Quite to the contrary, however, Appellants point out that, rather than calling into question Appellants' diagnostic approach, Kahn recognizes that significant parallels exist between *C. elegans* and mammalian insulin signaling. Kahn therefore *supports* the validity of Appellants' scientific discovery and the efficacy of the presently claimed diagnostic technique. Reliance by the Office on Kahn should be withdrawn.

With respect to the final reference, Ogg, the Office asserts (page 8):

> The state of the art a[t] the time of filing was such that it has been unclear whether the PTEN (daf-18) activity is regulated during insulin-like signaling or any other signaling activity, since PTEN lipid phosphatase activity is low in vitro due to a missing modification of the insulin signaling cascade.

As an initial matter, Appellants note that this statement inadvertently mischaracterizes the teachings of Ogg. The section of this reference relied on by the Office actually reads (page 891, top of right column):

> One attractive possibility is that the AKT kinases or PDK1 activates DAF-18 as a component in the recovery from an episode of insulin signaling. It <u>may</u> <u>be</u> significant that PTEN lipid phosphatase activity is low in vitro, <u>perhaps</u> due to a

> missing modification by the insulin signaling cascade.
> (emphasis added; citation omitted)

At this passage, Ogg is simply providing a possible reason for why others have observed low PTEN phosphatase activity *in vitro*. The reference is not questioning the role of DAF-18 in the insulin cascade.

Moreover, the Office's concern regarding the role of *daf-18*, or its mammalian counterpart, PTEN, in insulin signaling is misplaced. Appellants' specification establishes a clear role for PTEN in mammalian glucose homeostasis. Appellants teach (page 117, lines 3-7):

> Reduction in PTEN activity would be expected to potentiate insulin and/or insulin-like growth factor signaling, but an increase in PTEN activity would be expected to cause insulin resistance downstream of the insulin receptor, the type observed in late onset diabetes.

Appellants disclose that results regarding the regulation of insulin signaling in *C. elegans* are relevant to mammalian insulin signaling.

> These results further endorse the congruence between the *C. elegans* and mammalian insulin signaling pathways, strongly supporting the contention that new genes identified in the *C. elegans* pathway also act in mammalian insulin signaling. In addition, we have also found that the *C. elegans* PTEN lipid phosphatase homologue, DAF-18, acts upstream of AKT in this signaling pathway. Thus, our molecular genetic analysis maps mammalian PTEN action to the insulin signaling pathway. (page 3, lines 9-15 of the specification)

Further, following Appellants' discovery, other scientific results in this field have

substantiated Appellants' early, important findings underlying the present claims. For example, following Appellants' filing of the present application, Butler et al. (*Diabetes* 51:1028-1034, 2002; hereafter "Butler;" Exhibit A) published evidence that PTEN functions in mammalian glucose homeostasis. In particular, Butler teaches (in the abstract):

> Systemic administration of PTEN ASO [antisense oligonucleotides] once a week in mice suppressed PTEN mRNA and protein expression in liver and fat by up to 90 and 75% respectively, and normalized blood glucose concentrations in *db/db* and *ob/ob* mice. Inhibition of PTEN expression also dramatically reduced insulin concentrations in *ob/ob* mice, improved the performance of *db/db* mice during insulin tolerance tests, and increased Akt phosphorylation in liver in response to insulin. These results suggest that PTEN plays a significant role in regulating glucose metabolism in vivo by negatively regulating insulin signaling. (emphasis added)

Further evidence for the role of PTEN in insulin signaling is provided by Stiles et al. (*Proc. Natl. Acad. Sci. USA* 101:2082-2087, 2004; hereafter "Stiles;" Exhibit B). Stiles, like Butler, demonstrates a role for PTEN in mammalian insulin signaling, just as taught in Appellants' specification. Stiles states (in the abstract):

> *Pten* liver-specific deletion causes enhanced liver insulin action with improved systemic glucose tolerance. Thus, deletion of *Pten* in the liver may provide a valuable model that permits the study of metabolic actions of insulin signaling in the liver.

Thus, later publications substantiate Appellants' discovery and reinforce the fact that

PTEN plays a role in insulin signaling and therefore provides a marker for diagnosis of glucose metabolism disorders.

Appellants note that this evidence was improperly dismissed by the Office in the final Office Action. On this issue, the Office states (page 9):

> Applicant's argument that Butler et al. ... teaches that PTEN modulates mammalian insulin signaling just as the applicant disclosed has been found unpersuasive because each patent application is examined on its own merits and is considered enabled in view of its own disclosure. The issue is not whether other application[s] support their claims but whether one supports its claims. (emphasis original; citation omitted)

A similar position was taken with respect to Stiles. Appellants respectfully request reconsideration on this matter. Contrary to the Office's assertion, the specification clearly teaches that PTEN expression or function can be used in diagnosing an impaired glucose tolerance condition, obesity, or a propensity thereto in a patient, as is presently claimed. Butler and Stiles simply confirm the role of PTEN in mammalian insulin signaling, and thereby support Appellants' position that the specification enables the present diagnostic claims.

Clearly, as taught in Appellants' specification, and as supported by post filing art, PTEN plays a role in mammalian glucose homeostasis and therefore provides a valuable diagnostic for impaired glucose tolerance conditions and obesity. Nothing in the references cited by the Office refutes this evidence, and this basis for the enablement

rejection should be reversed.

II. Enablement of Diagnostic Methods

The enablement rejection, as set forth in the final Office Action and the Advisory Action, is also based on the assertion by the Office that Appellants' specification fails to enable one skilled in the art to make and use the presently claimed method. This basis for the rejection is applied in error.

The test of enablement is "whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with the information known in the art without undue experimentation." *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 802 F.2d. 1318, 231 U.S.P.Q. 81 (Fed. Cir. 1985). The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. In the present case, the claimed method requires only that one be able to analyze the level of PTEN expression or activity in a sample and compare that level to a control sample, with an increase in PTEN expression or activity indicating an impaired glucose tolerance condition, obesity, or a propensity thereto. Appellants' specification enables this method.

On this point, the Board is directed to the specification at page 117, lines 3-7, where it is taught that a reduction in PTEN activity potentiates insulin and/or insulin-like

growth factor signaling, whereas an increase in PTEN activity causes insulin resistance downstream of the insulin receptor. Thus, to diagnose an impaired glucose tolerance condition or obesity, one needs merely to determine if a patient exhibits increased PTEN levels.

Methods for analyzing the level of PTEN activity are described, for example, at page 119, lines 2-5, of the specification. There, Appellants teach that DAF-18/PTEN activity may be identified using a published *in vitro* lipid phosphatase assay as described by Maehama (*J. Biol. Chem.* 237:13375-13378, 1998). Alternatively, methods for analyzing PTEN expression levels are described, for example, at pages 150-152, where Appellants teach that antibodies to DAF polypeptides (of which PTEN is a member) are useful in immunological assays to detect expression in a patient sample; at pages 176 and 177, where Appellants teach that any DAF promoter may be fused to a reporter and used to monitor expression; and at page 195, lines 10-25, where Appellants teach methods of analyzing the expression of DAF nucleic acid and amino acid sequences, such as PTEN. These assays each determine PTEN levels, teaching how to carry out the presently claimed diagnostic method.

In the final Office Action, the Office also rejected the present claims based on the assertion that "it is unclear what would be a representative control sample" for diagnosing an increase in PTEN expression or activity. Appellants note, as set forth in the October 19, 2004 reply, that an appropriate control would be one obtained from an individual who

does not have an impaired glucose tolerance condition, is not obese, and does not have a propensity for acquiring these conditions. Such controls are referred to in Appellants' specification, for example, at page 126, lines 10-12, where Appellants teach that the appropriate controls for studies of insulin regulation are wild-type control mammals. Choosing an appropriate control is therefore addressed in Appellants' specification (and is consistent with standard assay conditions used in the art), and could readily be accomplished by a skilled artisan carrying out the presently claimed methods.

As a further basis for the enablement rejection, the Office, at page 10 of the final Office Action states:

> [A]nalyzing any and all kind of PTEN lipid phosphatase activity in any and all tissue sample[s] is not considered routine in the art.

On this point, Appellants first note that, as pointed out above, the specification teaches a method of determining PTEN lipid phosphatase activity that is standard in the art. This method can be used to carry out the presently claimed method and, therefore, enables this aspect of the claimed method. The enablement standard does not require Appellants to enable any and all PTEN lipid phosphatase assays.

Moreover, one skilled in the art would clearly compare tissues that normally express PTEN. As noted above, analyzing the level of PTEN expression is taught, for example, at pages 150-152 of Appellants' specification. Using this technique, one skilled in the art could readily determine which tissues (such as fat) should be compared. One

skilled in the art need not compare PTEN lipid phosphatase activity in any and all tissues; no undue experimentation is required to select a tissue sample.

Finally, as noted in the final Office Action and in the Advisory Action, the Office also maintains this rejection based on the assertion that "the specification as filed fails to provide a single working example, which establishes that PTEN modulates mammalian insulin signaling and/or is associated with the development of obesity." On this point, Appellants take issue with the standard applied by the Office. The standard for enablement is whether one reasonably skilled in the art could make or use the invention from the disclosure in the patent application coupled with the information known in the art without undue experimentation. Appellants' specification teaches that PTEN plays a role in mammalian glucose homeostasis, a finding that was confirmed by others after the application was filed. As pointed out above, Appellants' specification also teaches how one skilled in the art can carry out the presently claimed diagnostic method. Thus, Appellants submit that undue experimentation is not required to make or use the presently claimed invention. Appellants' specification meets the enablement standard, regardless of whether or not it contains a specific working example.

III. Scope of the Claims

As a final basis for the enablement rejection set forth in the April 19, 2004 Office Action, the Office asserts that "it is unclear how one skill[ed] in the art would diagnose

[an] impaired tolerance condition or propensity thereto by analyzing PTEN lipid phosphatase activity alone in type-I diabetic patients, wherein the impaired glucose tolerance is the result of loss of insulin secretion."

This basis for limiting the scope of the claims is unwarranted. Analyzing the level of PTEN expression or activity, for example, by measuring PTEN lipid phosphatase activity is workable even in the absence of insulin. As is noted above, the assay involves comparing the PTEN lipid phosphatase activity in a sample isolated from a patient with the level of PTEN in a control sample. Appellants' specification, for example, at page 108, lines 15-17, teaches that "PTEN has lipid phosphatase activity that dephosphorylates position 3 on the inositol ring of PIP_3 *in vitro* and decreases the level of the lipid products of PI3K in response to insulin signaling in human 293 cells." As insulin signaling is regulated by PTEN lipid phosphatase activity, in the absence of insulin signaling, i.e., even in the absence of insulin, the level of PTEN lipid phosphatase activity would clearly be different than in insulin's presence. Thus, one carrying out the claimed method would observe a difference in PTEN expression or activity between the sample from a patient who does not secrete insulin (e.g., a type I diabetic) and the control. This final basis for the rejection under 35 U.S.C. § 112, first paragraph should also be reversed.

Conclusion

Appellants respectfully request that the rejection of claims 1 and 3 be reversed. Enclosed is a check for $250 in payment of the fee required by 37 C.F.R. § 41.20(b)(2). Also enclosed is a petition to extend the period for filing an Appeal Brief pursuant to the October 21, 2004 Notice of Appeal for five months to May 23, 2005, as May 21st falls on a Saturday, and a check in the amount of the required fee. If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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Claims Appendix

1. A method of diagnosing an impaired glucose tolerance condition, obesity, or a propensity thereto in a patient, said method comprising analyzing the level of PTEN expression or activity in a sample isolated from said patient, whereby an increase in said level of PTEN expression or activity relative to a control sample is an indication of an impaired glucose tolerance condition, obesity, or a propensity thereto.

3. The method of claim, wherein said level of PTEN expression or activity is analyzed by measuring PTEN lipid phosphatase activity.

Evidence Appendix

Exhibit A: Butler et al., *Diabetes* 51:1028-1034, 2002 (copy attached).

Exhibit B: Stiles et al., *Proc. Natl. Acad. Sci. USA* 101:2082-2087, 2004 (copy attached).

EXHIBIT A

Specific Inhibition of PTEN Expression Reverses Hyperglycemia in Diabetic Mice

Madeline Butler, Robert A. McKay, Ian J. Popoff, William A. Gaarde, Donna Witchell, Susan F. Murray, Nicholas M. Dean, Sanjay Bhanot, and Brett P. Monia

Signaling through the phosphatidylinositol 3′-kinase (PI3K) pathway is crucial for metabolic responses to insulin, and defects in PI3K signaling have been demonstrated in type 2 diabetes. PTEN (MMAC1) is a lipid/protein phosphatase that can negatively regulate the PI3K pathway by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate, but it is unclear whether PTEN is physiologically relevant to insulin signaling in vivo. We employed an antisense oligonucleotide (ASO) strategy in an effort to specifically inhibit the expression of PTEN. Transfection of cells in culture with ASO targeting PTEN reduced PTEN mRNA and protein levels and increased insulin-stimulated Akt phosphorylation in α-mouse liver-12 (AML12) cells. Systemic administration of PTEN ASO once a week in mice suppressed PTEN mRNA and protein expression in liver and fat by up to 90 and 75%, respectively, and normalized blood glucose concentrations in *db/db* and *ob/ob* mice. Inhibition of PTEN expression also dramatically reduced insulin concentrations in *ob/ob* mice, improved the performance of *db/db* mice during insulin tolerance tests, and increased Akt phosphorylation in liver in response to insulin. These results suggest that PTEN plays a significant role in regulating glucose metabolism in vivo by negatively regulating insulin signaling. *Diabetes* 51:1028–1034, 2002

Phosphatidylinositol 3′-kinase (PI3K) is a crucial signaling enzyme whose activity is regulated by a variety of biological stimuli, including insulin (1). PI3K is composed of two subunits: the p85 regulatory subunit, containing two Src homology-2 domains, and the p110 catalytic subunit (2–4). Binding of insulin to its receptor activates the insulin receptor tyrosine kinase, resulting in autophosphorylation and phosphorylation of several substrates, including insulin receptor substrate (IRS)-1 through -4. IRS then binds to the regulatory subunit of PI3K through its Src homology domains, and this interaction activates the catalytic unit. Activated PI3K phosphorylates the 3′-position of the ring in inositol phospholipids, generating phosphatidylinositol (3,4), diphosphate, and phosphatidylinositol (3,4,5)-triphosphate (PIP3). The lipid products of PI3K initiate phosphorylation and activation of Akt, which is believed to act as a downstream mediator of many of the metabolic effects of insulin (5,6). Thus, the expression of inactive PI3K mutants or chemical agents, such as wortmannin and LY294002 that interfere with PI3K activity, inhibit Akt phosphorylation, glucose uptake, and glycogen and lipid synthesis in vitro (7,8).

PTEN (MMAC1/TEP1) is a dual-specificity protein phosphatase involved in signal transduction and tumor suppression (9,10). PTEN also has phosphoinositide 3′-phosphatase activity and is therefore capable of suppressing PI3K signaling by dephosphorylating PIP3 (11,12). Mutations in PTEN have been associated with several human cancers, and mice heterozygous for the PTEN gene have a high incidence of spontaneous tumors (13,14). Tumor cells and fibroblasts deficient in PTEN have elevated levels of PIP3 and phosphorylated Akt/protein kinase B (PKB) and are resistant to many apoptotic stimuli (15,16).

Because many of the metabolic effects of insulin are mediated through activation of PI3K and the subsequent rise in intracellular PIP3 concentrations, inhibition of a negative regulator of this pathway may enhance insulin signaling. Although the tumor-suppressive functions of PTEN have been elucidated, its physiological role in glucose metabolism in vivo is largely unknown. Inhibition of the *daf-18* gene, a homolog of PTEN in *Caenorhabditis elegans*, can partially bypass the need for DAF-2, an insulin receptor-like molecule (17,18). PTEN overexpression in vitro inhibits glucose uptake and GLUT4 transport in 3T3L1 cells, whereas microinjection of PTEN antibodies increased GLUT4 translocation (19). These results suggest that PTEN may modulate insulin signaling in vivo; however, the lethality of the PTEN null mutation has made this difficult to study. We therefore designed and characterized antisense oligonucleotides (ASOs) targeting PTEN and used them in vitro and in vivo to determine whether the inhibition of PTEN expression affects insulin signaling and glucose metabolism.

RESEARCH DESIGN AND METHODS

Oligonucleotides. A total of 80 oligonucleotides were screened for their ability to inhibit PTEN mRNA expression in T-24 bladder carcinoma cells by quantitative real-time RT-PCR. All oligonucleotides were synthesized as uniform phosphorothioate chimeric oligonucleotides, with 2′-*O*-methoxyethyl groups on bases 1–5 and 16–20. The oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer (Perkin Elmer-Applied Biosystems) and purified as described (20). Two active PTEN ASOs complementary to human and mouse PTEN mRNA (Genbank accession nos. AA017584 and AA124728, respectively), a six-base mismatch, and a control

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AS1, antisense oligonucleotide 1; ASO, antisense oligonucleotide; DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; IRS, insulin receptor substrate; MIS, mismatch control oligonucleotide; PI3K, phosphatidylinositol 3′-kinase; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PKB, protein kinase B; UC, universal control oligonucleotide.

oligonucleotide were used in the experiments described and are designated as follows: antisense oligonucleotide 1 (AS1) (ISIS 116847: 5′-CTGCTAGCCTCT GGATTTGA-3′, beginning at position 2097 in the human RNA); AS2 (ISIS 116845: 5′-CACATAGCGCCTCTGACTGGG-3′, beginning at position 1,539); MIS (ISIS 116848: 5′-CTTCTGGCATCCGGTTTAGA-3′, a six base mismatch to AS1); and UC, a universal control (ISIS 29848: synthesized using a mix of random mixture of A, G, T, and C so that the resulting preparation represents an equimolar mixture of all possible four [19] oligonucleotides).

Cell culture. 3T3L1 murine fibroblasts (American *Type Culture* Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (growth media). Cells were grown to confluence in 12- or 24-well plates before initiating differentiation. Confluent monolayers were differentiated to the adipocyte phenotype by culturing with 500 μmol/l isobutulmethylaxanthine, 250 nmol/l dexamethasone, and 400 nmol/l insulin in growth media for 3 days, followed by growth media alone for 3 days. Following this protocol, >90% of the adipocytes express the fully differentiated phenotype by 6 days after initiation.

Fully differentiated 3T3L1 adipocytes were transfected by the addition of serum-free DMEM and FuGENE6 (Roche) following the manufacturers instructions. The final concentration of 500 nmol/l oligonucleotide and a ratio of 4 μl FuGENE6 per microgram oligonucleotide were empirically determined to maximally suppress target RNA expression. Cell media was typically refreshed 36 h after transfection.

AML12 cells (American *Type Culture* Collection), a nontransformed hepatocyte cell line from transforming growth factor-β transgenic mice, were used to demonstrate antisense-mediated PTEN protein reduction and insulin-stimulated Akt phosphorylation in vitro. The cells were maintained in 90% of 1:1 mixture of DMEM and Ham's F12 medium containing 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, and 10% fetal bovine serum. The cells were treated with AS1 or mismatch control oligonucleotide (MIS) for 72 h using Lipofectin (Gibco) as a transfection agent per the manufacturer's instructions. Because AML12 cells require insulin for maintenance, the cells were serum- and insulin-starved for 8 h after transfection, and then 100 nmol/l insulin was added for 30 min before harvesting in lysis buffer for Western blotting.

Northern blots. RNA was prepared from cultured cells using a Qiagen RNA Easy Kit and from animal tissues homogenized in guanidinium isothiocyanate followed by cesium chloride gradients (21). Northern blots were performed as described using full cDNA probes generated by RT-PCR (22). The RNA signal was detected using a PhosphorImager (Molecular Dynamics) and normalized against the signal for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) using ImagePro software.

Western blots. Cells or tissues were harvested in lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris, pH 7.5, 1% Triton X-100, 0.5% NP-40, 0.25% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l ortho-vanadate, 1 mmol/l NaF, and 1:200 dilution of protease cocktail III; (Calbiochem), and the proteins were separated by SDS-PAGE. After transfer of the proteins onto polyvinylidine fluoride membranes, the blots were reacted with antibodies to phospho-Akt (New England Biolabs), Akt (New England Biolabs), or PTEN and developed using enhanced chemiluminescence (Amersham Pharmacia).

Mice and metabolic measurements. All animal experiments were performed under the institutional American Association for the Accreditation of Laboratory Animal Care (AALAC) guidelines. Male *db/db* mice (C57BLKS/J-*m* +/+*Lepr*^{db}) and age-matched lean littermates (C57BLKS/J-*m*+/+ *Lepr*^{db}) at 10 weeks of age or male *ob/ob* (C57BL/6J-*Lep*^{ob}) at 8 weeks of age (The Jackson Laboratory) were used for all experiments. Mice were maintained on a 12-h light/dark cycle and fed ad libitum unless otherwise noted. Whole blood was obtained from the retro-orbital sinus of fed mice, and glucose was measured using a Metabolics glucose oxidase-based analyzer. Mice were weighed once a week, and food intake was monitored over a 24-h period. An insulin tolerance test was performed after a 4-h fast by intraperitoneal injection of 1 unit/kg human insulin (Lilly). Blood was drawn from the tail before insulin injection (time 0) and then 30, 60, and 90 min afterward and measured as described above. For in vivo phospho-Akt measurements, mice were fasted for 12 h and then injected with 2 units/kg insulin.

Serum glucose, triglycerides, and cholesterol concentrations were analyzed on a Johnson and Johnson Vitros 950 automated clinical chemistry analyzer, and serum insulin concentrations were quantitated using an enzyme-linked immunosorbent assay for rat insulin (Alpco).

RESULTS

Characterization of PTEN ASO in vitro. ASOs designed to be complementary to human and mouse PTEN genomic sequences were screened for suppression of PTEN mRNA expression in cells in vitro, as previously described (23). The most potent oligonucleotide from the screen, AS1 (see RESEARCH DESIGN AND METHODS), reduced PTEN mRNA levels in a concentration-dependent manner in 3T3-L1 adipocytes. Similar results were obtained with a second PTEN antisense (AS2) that hybridizes to a different region of the PTEN mRNA. A control antisense with six mismatched nucleotides (MIS) to AS1 was inactive in reducing PTEN mRNA expression. Maximal inhibition (90%) of PTEN protein expression was achieved after 72 h of oligonucleotide treatment, which is presumably indicative of the intrinsic half-life of the PTEN protein (data not shown).

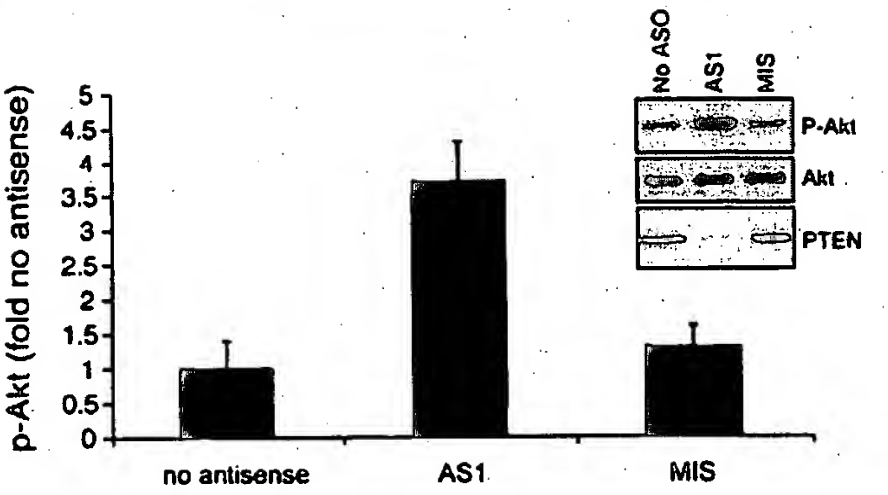

FIG. 1. Reduction of PTEN protein levels and stimulation of Akt phosphorylation by PTEN antisense treatment in vitro. AML12 cells were treated with AS1 or MIS for 72 h using Lipofectin as a transfection agent. Immunoblots of cell proteins were sequentially reacted with antibodies to phospho-Akt, -Akt, and -PTEN. Data are expressed as the fold increase in phospho-Akt intensity compared with Lipofectin-only treated cells. Graphs represent the mean of three replicates ±SD.

As described above, PIP3 is believed to initiate phosphorylation and activation of Akt, an important downstream mediator of the metabolic effects of insulin (24). We reasoned that if PTEN is indeed involved in modulating insulin signaling, inhibition of PTEN expression might increase the level of Akt phosphorylation in response to insulin. To test this, AML12 cells were treated with PTEN AS1, and the effects on PTEN protein levels and insulin-stimulated Akt phosphorylation were examined. Cells treated with PTEN AS1 resulted in a >90% reduction in PTEN protein levels (Fig. 1). Furthermore, PTEN AS1 treatment resulted in an increase in insulin-stimulated phosphorylation of Akt by ~3.5-fold, relative to untreated and MIS-treated cells (Fig. 1), whereas the Akt protein levels remained the same.

Antisense-mediated inhibition of PTEN expression in vivo. Based on the in vitro results obtained with AS1, we reasoned that inhibition of PTEN expression might improve insulin sensitivity in the *db/db* mouse, a rodent model of type 2 diabetes. First, we investigated the ability of systemically administered AS1 to reduce PTEN mRNA and protein levels in insulin-sensitive tissues. The half-life of 2′-*O*-methoxyethyl chimeric phosphorothioate oligonucleotides is ~7 to 19 days in the liver, depending on the dose (25). Therefore, *db/db* mice were treated by intraperitoneal injection once a week for 4 weeks with 10, 25, or 50 mg/kg of AS1, and PTEN mRNA levels in liver, fat, and muscle tissues were measured by Northern blotting. PTEN mRNA levels were reduced in a dose-dependent manner in

liver extracts from treated mice relative to saline controls, with maximal inhibition occurring (88%) at the 50-mg/kg dose (Fig. 2*A*). In lean littermates dosed with 100 mg/kg of AS1, PTEN mRNA levels were also reduced by >90%, relative to saline-treated controls. There was no apparent difference in the relative levels of PTEN mRNA in untreated lean versus *db/db* mice. Moreover, neither the MIS nor another control oligonucleotide, universal control oligonucleotide (UC), affected PTEN mRNA levels significantly. Also, the mRNA levels of PTP1B and SHIP2, two other phosphatases that have the potential to inhibit insulin signaling (26,27), were not affected by PTEN AS1 treatment (Fig. 2*B*). These results demonstrate that the effect of AS1 was both PTEN target–specific and antisense sequence–specific and indicate that the metabolic effects of the PTEN antisense (described below) were primarily caused by a specific reduction in PTEN expression.

PTEN protein levels in liver samples from saline-, AS1-, and UC-treated *db/db* mice were analyzed by Western blotting. After 4 weeks of AS1 treatment, a dose-dependent decrease in PTEN protein levels in livers from *db/db* mice was observed (Fig. 2*C*). Reduction of PTEN protein levels was also observed in livers from lean littermates treated with AS1. As with the mRNA results, no difference in the relative levels of PTEN protein in control lean versus *db/db* mouse livers was apparent, nor were any effects observed by a control oligonucleotide (UC) on PTEN protein levels.

Northern analysis of other insulin-sensitive tissues demonstrated that PTEN mRNA levels were also reduced in a dose-dependent manner, relative to saline controls, in fat tissue from AS1-treated *db/db* mice, with maximal inhibition of 80% at the 50 mg/kg dose (Fig. 2*D*). A similar reduction in PTEN protein levels in fat from AS1-treated mice was also observed (data not shown). PTEN message levels appeared to be less abundant in muscle relative to liver and fat, and no consistent reduction in PTEN mRNA expression was observed in the skeletal muscle of animals treated with PTEN AS1. This result is in agreement with pharmacokinetic studies showing that accumulation of oligonucleotides in muscle after parenteral injection is relatively low (28). Interestingly, no PTEN protein was detectable on immunoblots of muscle lysates (data not shown).

Effect of inhibiting PTEN expression on glucose, insulin, and lipid concentrations in diabetic and lean mice. Having characterized the effect of AS1 on PTEN mRNA and protein expression in vivo, we next investigated the effect of inhibiting PTEN expression on hyperglycemia in *db/db* and *ob/ob* mice. Blood glucose concentrations in *db/db* mice were reduced in a dose-dependent manner over the course of a 4-week treatment with AS1 (Fig. 3*A*), becoming normalized (138 ± 5 mg/dl) at the highest dose tested (50 mg/kg). The second PTEN antisense, AS2, produced a similar reduction in serum glucose levels at the end of 4 weeks of treatment (Fig. 3*B*). In related studies, treatment of *db/db* mice with PTEN AS2 resulted in a reduction of PTEN mRNA and protein levels in liver that was comparable with that produced in animals treated with PTEN AS2 (data not shown). In contrast, neither the MIS nor UC controls affected glucose levels. Furthermore, PTEN antisense treatment had no effect on glucose concentrations in lean littermates, despite the fact

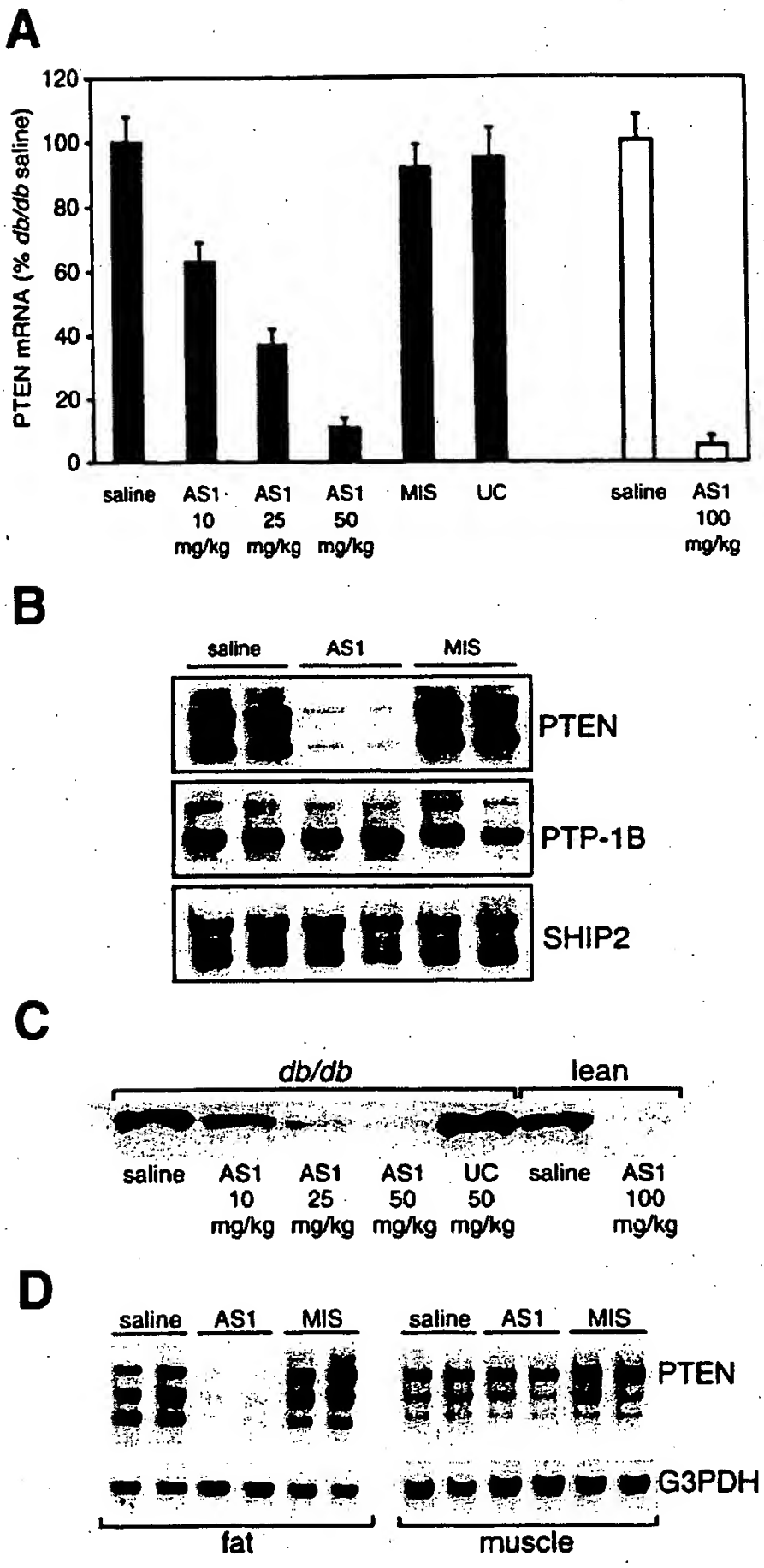

FIG. 2. PTEN antisense specifically reduces PTEN mRNA and protein levels in livers and fat from *db/db* mice. *A*: Dose-dependent reduction of PTEN mRNA levels in liver. *db/db* (black bars) and lean (white bars) mice received indicated doses of ASOs intraperitoneally once a week for 4 weeks. Total mRNA was prepared from liver and was analyzed by Northern blotting (n = 3 per group). The PTEN signal was normalized against the signal for G3PDH. Data are expressed as the mean percentage of mRNA levels in saline-treated *db/db* mice ±SD. *B*: Specificity of PTEN antisense. Representative Northern blots of PTEN mRNA (*A*), PTP1B mRNA (*B*), and SHIP2 mRNA (*C*) in livers from *db/db* mice treated once a week for 4 weeks with saline, 50 mg/kg AS1, or 50 mg/kg MIS. Each lane contained 25 μg of RNA from an individual animal. *C*: Reduction of PTEN protein expression in liver. PTEN immunoblots of proteins in liver lysates from mice treated for 4 weeks with indicated doses of AS1 or UC. Each lane contained 50 μg of protein. *D*: Reduction of PTEN mRNA in fat but not muscle. Representative Northern blots of PTEN and G3PDH mRNA in fat and muscle from *db/db* mice treated with saline, 50 mg/kg AS1, or 50 mg/kg MIS.

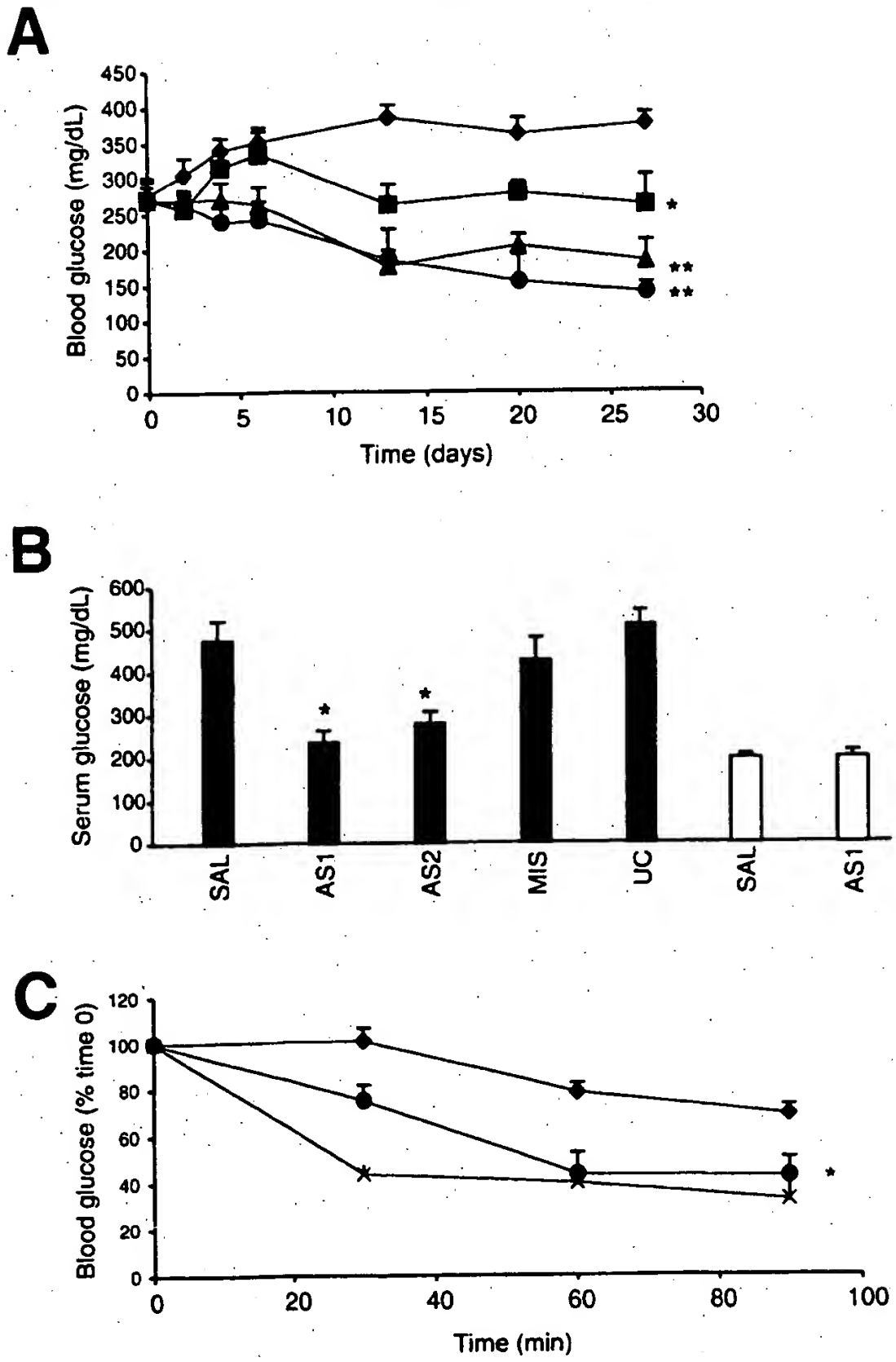

FIG. 3. Inhibition of PTEN expression lowers glucose and increases insulin sensitivity in *db/db* mice. *A*: Time- and dose-dependence of glucose-lowering effects of PTEN AS1. *db/db* mice were injected intraperitoneally once a week with saline (♦) or 10 mg/kg (■), 25 mg/kg (▲), or 50 mg/kg(●) of AS1 in saline. Mice were bled every 2 days for the first week and then once a week thereafter, 6 days after the previous dose. Values are expressed as means ± SE (n = 6–8). Statistical analysis was done using ANOVA repeated measures followed by Bonferroni-Dunn. Compared with saline-treated, **$P < 0.0001$, *$P < 0.01$. *B*: Specificity of the antidiabetic effect of PTEN ASOs on serum glucose concentrations. *db/db* mice (■, n = 5–6) were injected intraperitoneally with 50 mg/kg of indicated oligonucleotides for 4 weeks. Lean littermates (□, n = 5) were dosed with saline or 100 mg/kg of AS1. Statistics were performed using ANOVA, followed by Bonferroni-Dunn. Compared with saline-treated, *$P < 0.005$. *C*: Insulin tolerance test in PTEN antisense–treated mice. Mice were treated once a week for 3 weeks with saline (♦) or 50 mg/kg AS1 (●) (n = 5). Lean controls (x) were untreated. Results are expressed as the mean ±SE percentage of the glucose concentration at time 0. Statistical analysis was done using ANOVA repeated measures followed by Bonferroni-Dunn. Compared with saline-treated, *$P < 0.05$.

that PTEN mRNA and protein levels were reduced to the same extent as that of *db/db* mice.

To determine whether inhibition of PTEN expression had an effect on insulin sensitivity, an insulin tolerance test was performed in *db/db* mice treated once a week for 3 weeks with saline or AS1 (Fig. 3*C*). PTEN AS1 significantly increased sensitivity to insulin; the relative blood glucose concentrations in the AS1-treated mice were significantly lower at all time points after insulin injection compared with those in saline-treated animals. Also, inhibition of PTEN expression did not appear to cause hypoglycemia, in that glucose levels in mice remained normoglycemic in PTEN AS1–treated *db/db* and lean mice fasted for 16 h (data not shown).

Inhibition of PTEN expression also lowered serum triglyceride and cholesterol concentrations in *db/db* mice in a dose-dependent manner (Table1). Lipid concentrations were unaffected relative to lean littermates at the 50-mg/kg dose of AS1, and the control oligonucleotide had no effect. Treated *db/db* mice gained significantly more weight than saline- and control antisense–treated *db/db* mice, despite the fact that food intake was similar in all groups. However, *ob/ob* mice did not gain weight relative to saline-treated animals during their 4-week antisense treatment (see below). No significant changes in body composition (i.e., lean versus fat body mass) were observed in any mice treated with PTEN ASOs (data not shown).

The effect of inhibiting PTEN expression on hyperinsulinemia was investigated in *ob/ob* mice, which have higher circulating levels of insulin and are less hyperglycemic than *db/db* mice. Male *ob/ob* mice were injected with 50 or 20 mg/kg of AS1 on day 0 and then with either 20 or 10 mg/kg a week thereafter for 3 weeks, a dosing schedule designed to attain more moderate steady-state levels of oligonucleotide in liver. At the end of 4 weeks, PTEN

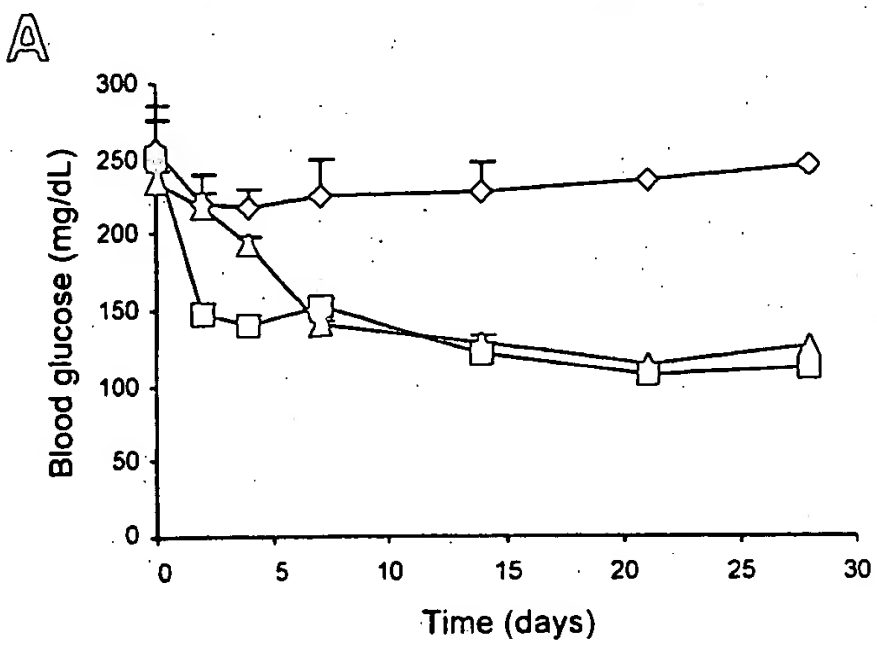

FIG. 4. Reduction of blood glucose (*A*) and serum insulin (*B*) in *ob/ob* mice treated with PTEN antisense. *A*: *Ob/ob* mice were injected intraperitoneally once a week with saline (◇) or 50 mg/kg AS1 on day 0, followed by 20 mg/kg once a week thereafter (▣), or 20 mg/kg AS1 on day 0, followed by 10 mg/kg once a week thereafter (△). Values are expressed as means ± SE (n = 8). *B*: *ob/ob* mice treated as described above were fasted for 4 h before measuring serum insulin after 2 weeks (□) and 4 weeks (□) of dosing. Values are expressed as the means ± SE (n = 6).

mRNA levels were reduced by 71% in the higher-dose group and by 49% in the lower-dose group (data not shown) relative to saline-treated animals, and blood glucose concentrations were normalized by 2 weeks in both dose groups (Fig. 4*A*). Serum insulin levels were reduced by 90% at 2 weeks in the higher-dose group and by 84% at 4 weeks in the lower-dose group (Fig. 4*B*).

Effect of inhibiting PTEN expression on Akt phosphorylation in diabetic and lean mice. If the effects of PTEN inhibition on glucose and insulin levels in diabetic mice are caused by an activation of PI3K signaling, evidence of a biochemical improvement in insulin signaling downstream of PI3K should be detectable. Because Akt activation is dependent on the products of PI3K, we reasoned that decreasing PTEN expression would result in increased levels of Akt phosphorylation in response to insulin in diabetic animals. To test this, *ob/ob* mice and their lean littermates were treated with either saline, AS1, or MIS at 50 mg/kg once a week for 2 weeks, and PTEN and phospho-Akt protein levels were determined in liver. As can be seen in Fig. 5*A*, PTEN protein expression was reduced by ~90% in livers from both lean and *ob/ob* mice treated with AS1, relative to saline-treated mice. In lean mice, neither the basal levels of Akt phosphorylation nor the sixfold increase in Akt phosphorylation in response to a bolus insulin injection were affected by PTEN antisense treatment (Fig. 5*B*). As has been previously reported in diabetic rats (29), no increase in Akt phosphorylation in response to insulin was observed in control-treated *ob/ob* mice. However, PTEN AS1 treatment appeared to restore Akt phosphorylation in response to insulin in *ob/ob* mice (Fig. 5*C*). However, PTEN AS1 treatment did not appear to affect basal levels (non–insulin-stimulated) of phosphorylated Akt in *ob/ob* mice.

DISCUSSION

The molecular defects that cause insulin resistance and hyperglycemia in type 2 diabetes have not been well defined. Impaired insulin receptor function leading to reduced activation of PI3K could be a cause of insulin resistance, or the primary defect may lie further downstream in the PI3K pathway. The results presented here indicate that PTEN, a tumor suppressor with phosphoinositide 3′-phosphatase activity, may play a role in glucose metabolism in vivo by negatively regulating insulin signaling.

Several lines of evidence indicate that PI3K activation and the subsequent rise in PIP3 concentrations are necessary for many of the metabolic responses to insulin, including Akt activation, glucose transport, and glycogen and lipid synthesis. PTEN is capable of dephosphorylating PIP3 (12), and cells in which PTEN activity has been inhibited have elevated PIP3 concentrations and higher levels of Akt phosphorylation (16). Thus, it seems logical that PTEN can regulate insulin signaling through the PI3K pathway. Indeed, Nakashima et al. (19) recently demonstrated that overexpression of PTEN in 3T3-L1 cells inhibits glucose uptake and GLUT4 translocation in vitro, whereas microinjection of a PTEN antibody increased basal and insulin-stimulated GLUT4 translocation also in vitro. Our results demonstrating that antisense-mediated reduction of PTEN expression increased insulin-dependent Akt phosphorylation in vitro also supports the conclusion that PTEN may negatively regulate insulin signaling in cultured cells.

Moreover, using our antisense approach, we were able to determine the effect of inhibiting PTEN expression in animals, and our results suggest that PTEN plays an important role in glucose homeostasis in vivo as well. We have shown that systemic administration of PTEN antisense reduced PTEN mRNA and protein expression in a dose-dependent manner in mouse liver but had no effect on the levels of the phosphatases PTP1B and SHIP2. Similar results have recently been obtained using a Fas ASO, which reduced Fas mRNA and protein expression in hepatocytes by up to 90% after systemic injection in mice (25). We also found that systemically administered PTEN oligonucleotides are capable of reducing target expression in fat, but not muscle, and that PTEN mRNA and protein levels are much less abundant in muscle than in fat and liver. Taken together, these results indicate that the effects of the antisense were indeed specific for PTEN and suggest that the reduction of PTEN expression primarily in

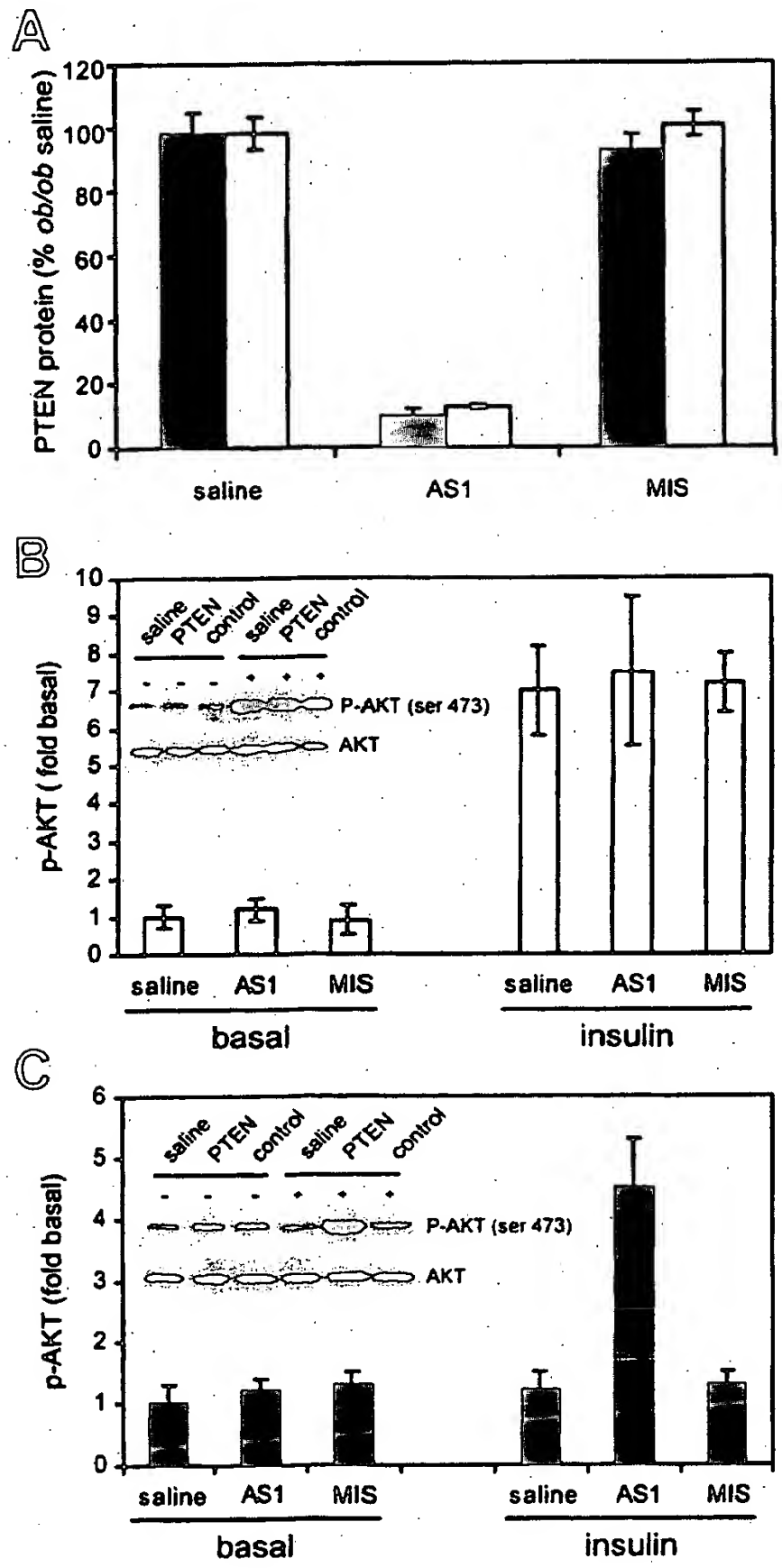

FIG. 5. Reduction of PTEN expression in *ob/ob* and lean mice and increased Akt phosphorylation in *ob/ob* mice treated with PTEN antisense. *A*: Reduction of PTEN protein levels in both *ob/ob* (▩) and lean (□) mouse livers after PTEN antisense treatment. Mice (n = 4 per group) were injected for 2 weeks with saline or 50 mg/kg ASI or MIS oligonucleotides, and liver lysates were immunoblotted with antibodies to PTEN and G3DPH. Data are expressed as the mean percentage of normalized PTEN protein levels in saline-treated *ob/ob* mice ±SD. *B* and *C*: Phospho-Akt levels in livers of lean and *ob/ob* mice without (basal) or 5 min after intraperitoneal injection of 2 units/kg of insulin. Immunoblots of proteins were sequentially reacted with antibodies to phospho-Akt and Akt. Data are expressed as the fold increase in phospho-Akt intensity compared with basal levels in saline-treated mice relative to Akt protein levels. Graphs represent the means ± SD. Representative gels of pooled samples (n = 3) from each group are also shown for lean and *ob/ob* mice.

liver, with some possible contribution in fat, was sufficient to produce the observed metabolic changes in diabetic mice. Improved insulin sensitivity in liver of diabetic mice would be expected to reduce hepatic glucose production and thereby reverse hyperglycemia.

Antisense-mediated inhibition of PTEN expression normalized glucose concentration in both *db/db* and *ob/ob* mice, improved insulin sensitivity in *db/db* mice, and lowered insulin concentrations dramatically in *ob/ob* mice. The fact that inhibition of PTEN expression reversed hyperglycemia and reduced insulin resistance in diabetic mice, without affecting glucose levels in lean mice, suggests that the reduction in PTEN expression compensated for a defect in the PI3K pathway in diabetic mice. Alternatively, it is possible that inhibition of PTEN in diabetic mice may somehow compensate for defects in other pathways that are unrelated to PI3K but may contribute to insulin resistance in these animals. No detectable difference in PTEN mRNA or protein levels in lean versus *db/db* mice was observed, so it does not appear that an increase in PTEN expression levels is the primary defect in these mice. It has previously been demonstrated that IRS-associated PI3K activity is decreased significantly in *ob/ob* mouse liver (30) and that Akt/PKB activity is reduced in liver and muscle from diabetic rats and humans (29). We have demonstrated that inhibition of PTEN expression in vivo restores insulin-stimulated Akt phosphorylation in *ob/ob* diabetic mice to a level comparable with that in lean mice. We have also observed significant increases in liver Akt phosphorylation in *db/db* mice treated with PTEN antisense (data not shown). Thus, it seems logical that a reduction in PTEN expression after antisense treatment resulted in increased PI3K activity by increasing the half-life and/or effective concentration of PIP3 produced during insulin activation. This logic is consistent with the putative role of PTEN in PI3K signaling, as well as with the results of other investigators who have employed different approaches for suppressing PTEN activity in vitro (11–14,17–19). Nevertheless, we cannot rule out the possibility that the effects of PTEN inhibition on Akt phosphorylation and insulin sensitivity that we have observed in vitro and in vivo may not be directly related to increased PI3K activity and increased PIP3 levels because neither of these end points were measured directly. Although PTEN antisense treatment had no effect on insulin-dependent Akt phosphorylation or on circulating glucose levels in lean mice, serum insulin levels were decreased by 50%, suggesting that PTEN inhibition may increase insulin sensitivity in lean mice as well. Interestingly, a recent report about a Cowden's disease patient with a heterozygous PTEN mutation indicated improved insulin sensitivity, as measured by glucose clearance and hyperinsulemic-euglycemic clamp (31). However, although several groups have demonstrated an increased incidence of tumors in PTEN heterozygous mice, no changes in blood glucose concentrations have been reported. This finding may not be surprising in view of the fact that in our studies, reducing PTEN expression by 90% had no significant effect on glucose levels in lean mice.

In conclusion, our results demonstrate that suppression of PTEN expression produces a marked improvement in blood glucose concentrations and insulin sensitivity in diabetic mice and suggest that pharmacological inhibition of negative regulators of the PI3K pathway may represent

a therapeutic approach for the treatment of type 2 diabetes.

REFERENCES

1. Alessi DR, Downes CP: The role of PI 3-kinase in insulin action. *Biochim Biophys Acta* 1436:151–164, 1998
2. Otsu M, Hiles I, Gout I, Fry MJ, Ruiz-Larrea F, Panayotou G, Thompson A, Dhand R, Hsuan J, Totty N, et al.: Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src complexes, and PI3-kinase. *Cell* 65:91–104, 1991
3. Escobedo JA, Navankasattusas S, Kavanaugh WM, Milfay D, Fried VA, Williams LT: cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF beta-receptor. *Cell* 65:75–82, 1991
4. Skolnik EY, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, Ullrich A, Schlessinger J: Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* 65:83–90, 1991
5. Martin SS, Rose DW, Saltiel AR, Klippel A, Williams LT, Olefsky JM: Phosphatidylinositol 3-kinase is necessary and sufficient for insulin-stimulated stress fiber breakdown. *Endocrinology* 137:5045–5054, 1996
6. Kohn AD, Summers SA, Birnbaum MJ, Roth RA: Expression of a constitutively active Akt Ser/Thr kinase in 3T3–L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem* 271:31372–31378, 1996
7. Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR: Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol* 14:4902–4911, 1994
8. Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M: Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes: studies with a selective inhibitor wortmannin. *J Biol Chem* 269:3568–3573, 1994
9. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R: PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer [see comments]. *Science* 275:1943–1947, 1997
10. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV: Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 15:356–362, 1997
11. Lee JO, Yang H, Georgescu MM, Di Cristofano A, Maehama T, Shi Y, Dixon JE, Pandolfi P, Pavletich NP: Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell* 99:323–334, 1999
12. Maehama T, Dixon JE: The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 273:13375–13378, 1998
13. Di Cristofano A, Kotsi P, Peng YF, Cordon-Cardo C, Elkon KB, Pandolfi PP: Impaired Fas response and autoimmunity in Pten+/- mice. *Science* 285: 2122–2125, 1999
14. Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE, Parsons R: Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc Natl Acad Sci U S A* 96:1563–1568, 1999
15. Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP, Tonks NK: The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc Natl Acad Sci U S A* 95:13513–13518, 1998
16. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW: Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 95:29–39, 1998
17. Ogg S, Ruvkun G: The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. *Mol Cell* 2:887–893, 1998
18. Mihaylova VT, Borland CZ, Manjarrez L, Stern MJ, Sun H: The PTEN tumor suppressor homolog in Caenorhabditis elegans regulates longevity and dauer formation in an insulin receptor-like signaling pathway. *Proc Natl Acad Sci U S A* 96:7427–7432, 1999
19. Nakashima N, Sharma PM, Imamura T, Bookstein R, Olefsky JM: The tumor suppressor PTEN negatively regulates insulin signaling in 3T3- L1 adipocytes. *J Biol Chem* 275: 12889–12895., 2000
20. Baker BF, Lot SS, Condon TP, Cheng-Flournoy S, Lesnik EA, Sasmor HM, Bennett CF: 2'-O-(2-methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. *J Biol Chem* 272: 11994–12000, 1997
21. Dean NM, McKay R: Inhibition of protein kinase C-alpha expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides. *Proc Natl Acad Sci U S A* 91:11762–11766, 1994
22. Condon TP: Generation of single-stranded DNA hybridization probes by PCR using a short, synthetic DNA template. *Biotechniques* 26:18–19, 1999
23. Li F, Ackermann EJ, Bennett CF, Rothermel AL, Plescia J, Tognin S, Villa A, Marchisio PC, Altieri DC: Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol* 1:461–466, 1999
24. Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT: Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B [see comments]. *Science* 277:567–570, 1997
25. Zhang H, Cook J, Nickel J, Yu R, Stecker K, Myers K, Dean NM: Reduction of liver fas expression by an antisense oligonucleotide protects mice from fulminant hepatitis. *Nat Biotechnol* 18:862–867, 2000
26. Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP: Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene [see comments]. *Science* 283:1544–1548, 1999
27. Erneux C, Govaerts C, Communi D, Pesesse X: The diversity and possible functions of the inositol polyphosphate 5-phosphatases. *Biochim Biophys Acta* 1436:185–199, 1998
28. Butler M, Stecker K, Bennett CF: Cellular distribution of phosphorothioate oligodeoxynucleotides in normal rodent tissues. *Lab Invest* 77:379–388, 1997
29. Nawano M, Ueta K, Oku A, Arakawa K, Saito A, Funaki M, Anai M, Kikuchi M, Oka Y, Asano T: Hyperglycemia impairs the insulin signaling step between PI 3-kinase and Akt/PKB activations in ZDF rat liver. *Biochem Biophys Res Commun* 266:252–256, 1999
30. Kerouz NJ, Horsch D, Pons S, Kahn CR: Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. *J Clin Invest* 100: 3164–3172, 1997
31. Iida S, Ono A, Sayama K, Hamaguchi T, Fujii H, Nakajima H, Namba M, Hanafusa T, Matsuzawa Y, Moriwaki K: Accelerated decline of blood glucose after intravenous glucose injection in a patient with Cowden disease having a heterozygous germline mutation of the PTEN/MMAC1 gene. *Anticancer Res* 20:1901–1904, 2000

EXHIBIT B

Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity

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In the liver, insulin controls both lipid and glucose metabolism through its cell surface receptor and intracellular mediators such as phosphatidylinositol 3-kinase and serine-threonine kinase AKT. The insulin signaling pathway is further modulated by protein tyrosine phosphatase or lipid phosphatase. Here, we investigated the function of phosphatase and tension homologue deleted on chromosome 10 (PTEN), a negative regulator of the phosphatidylinositol 3-kinase/AKT pathway, by targeted deletion of *Pten* in murine liver. Deletion of *Pten* in the liver resulted in increased fatty acid synthesis, accompanied by hepatomegaly and fatty liver phenotype. Interestingly, *Pten* liver-specific deletion causes enhanced liver insulin action with improved systemic glucose tolerance. Thus, deletion of *Pten* in the liver may provide a valuable model that permits the study of the metabolic actions of insulin signaling in the liver, and PTEN may be a promising target for therapeutic intervention for type 2 diabetes.

Insulin controls glucose and lipid homeostasis by modulating the function of multiple organs, including liver, muscle, and fat. In muscle and fat, insulin stimulates glucose uptake, resulting in glucose clearance from circulation. In liver, insulin promotes glycogen synthesis and glycolysis, as well as fatty acid (FA) synthesis. Impairment of the insulin signaling pathway plays a key role in the development of type 2 diabetes (T2D). Knockout and transgenic studies of molecules in this pathway have provided novel insights into the understanding of the molecular mechanisms underlying T2D (1). The muscle insulin receptor (IR) knockout model demonstrated normal insulin levels, in association with a metabolic syndrome characterized by defective FA metabolism (2). The liver IR knockout mice displayed severe insulin resistance and mild diabetes, in conjunction with hyperinsulinemia (3). Similar gene knockout studies were also performed with glucose transporter type-4 in muscle and fat (4, 5). Together with earlier studies (6) on murine models of obesity, these knockout studies revealed the importance of cooperation among different organs in the regulation of glucose and FA homeostasis (7), as well as the vital role liver plays in this collaboration.

The importance of phosphatidylinositol 3-kinase (PI3-kinase)/AKT in insulin signal has been suggested by both molecular and genetic studies (8–10). Insulin signal leads to the activation of PI3-kinase and its downstream target, AKT. One of the negative regulators of the insulin signaling pathway is PTEN (phosphatase and tension homologue deleted on chromosome 10), a lipid and protein phosphatase (11). In C-elegans, the PTEN homolog, DAF-18, acts in insulin receptor-like pathway and regulates longevity and dauer larva development (12–15). Hyperactivation of the PI3-kinase/AKT pathway also appears to be a main result of *Pten* deletion in mammalian systems (16). Unfortunately, *Pten*-deficient mice die at early stage of embryonic development (17–20), which precludes study of the role of PTEN in the mammalian insulin signaling pathway in a whole-animal setting. Injection of antisense oligonucleotide has demonstrated that inhibiting *Pten* may improve the glycemic control in *ob/ob* and *db/db* mice (21). In this study, we have generated an animal model by disrupting the *Pten* gene in mouse liver to assess the biological functions of PTEN in insulin signaling and the development of insulin resistance.

Materials and Methods

Animals. $Pten^{loxP/loxP}$ mice (22) were bred with Alb-Cre mice to generate mice with liver-specific deletion (23). All experiments were conducted in accordance with University of California, Los Angeles live animal welfare guidelines.

Animals 1, 3, and 6 months old were used for the experiments. For metabolic measurements, animals were fasted overnight. Blood samples were taken next morning for assessment of glucose, insulin (Linco and Alpco), nonesterified FAs (NEFA) (Wako), leptin (Crystal Chem) and triglyceride (TG) (Thermo DMA) by using manufactured kits. For tissue collection, mice were fasted overnight and blood was collected from cardiac puncture. Livers were perfused and collected in formalin for histology or in TRIzol (Invitrogen) for RNA. Extra liver tissues were flash-frozen in liquid nitrogen for protein analysis or frozen section.

Glucose Tolerance Test (GTT). A GTT was performed on mice that were fasted for 16 h. For glucose measurement, tail veins were punctured and a small amount of blood was released and applied onto a Therasense glucometer. For GTT, mice were given a single dose (2 g/kg of body weight) of D-dextrose (Sigma) by i.p. injection after a baseline glucose check. Circulating glucose levels were then measured at indicated time points after glucose injection.

Analysis of Long-Chain FA Uptake by Hepatocytes. Primary hepatocytes were prepared by two-step perfusion with liver perfusion and digestion medium, according to the manufacturer's instructions (Invitrogen), and were immediately used for uptake assays. Fluorescence-activated cell sorter-based short-term (30 sec) FA uptake assays were performed as described (24).

Analysis of FA Synthesis Rate By Using GC-MS. Deuterium water (D_2O; Aldrich) was provided as deuterium source for incorporation into the FA in *de novo* lipogenesis (25, 26). An initial priming dose of deuterium water (4% body weight) by i.p. injection was followed by a maintenance dose of 6% (vol/vol)

Abbreviations: FA, fatty acid; NEFA, nonesterified FA; T2D, type 2 diabetes; PI3-kinase, phosphatidylinositol 3-kinase; PTEN, phosphatase and tension homologue deleted on chromosome 10; TG, triglyceride; GTT, glucose tolerance test; PTP, protein tyrosine phosphatase; GS, glycogen synthase; PEPCK, phospho*enol*pyruvate carboxykinase; G6Pase, glucose-6-phosphatase.

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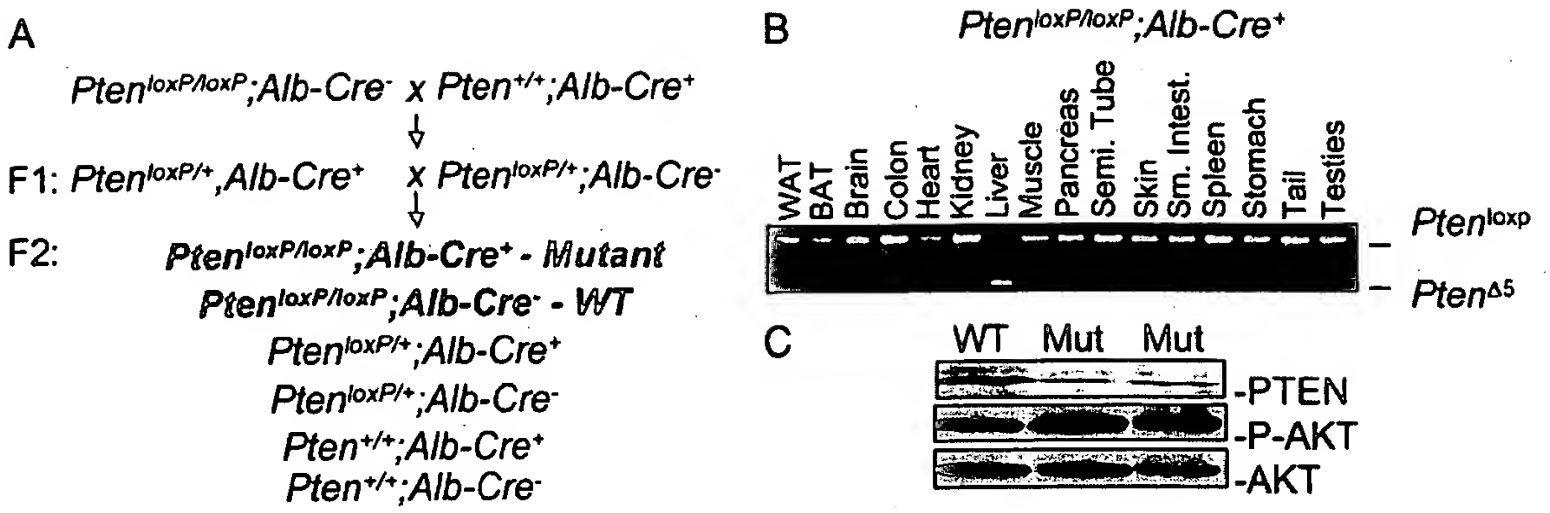

Fig. 1. Liver-specific deletion of *Pten*. (*A*) Breeding strategy. (*B*) Liver-specific deletion of the *Pten* gene. PCR analysis of DNA from different tissues of $Pten^{lox/lox};Alb\text{-}Cre^{+}$ mice. (*C*) Western analysis for PTEN (*Top*), *p*-AKT (*Middle*) and AKT (*Bottom*).

D_2O in drinking water. Plasma samples were collected from orbital eye bleeding before and at 2, 4, 7, and 9 days after D_2O injection. At the end of the experiment, tissue samples were collected from perfused livers after overnight fasting. Total lipid was isolated from both plasma and liver samples after saponification. The isolated FAs were methylated and injected onto GC/MS with C-18 column. The spectrum of the palmitate peak (270–276 *m/z*) was analyzed for its isotopomer distribution and deuterium content, which were used to calculate the fraction of newly synthesized FA (25, 26).

Assessment of Lipid Secretion Rate *in Vivo*. Overnight-fasted mice were injected with 10% tyloxapol (5 μl/g body weight) through the tail vein to coat the lipoprotein particles. Plasma was collected through orbital eye bleeding before and 30, 60, and 90 min after tyloxapol injection, and TG levels were determined (27).

Western Blot Analysis. Liver samples were homogenized in PBS (pH 74)/1% NP-40/0.5% sodium deoxycholate/0.1% SDS containing protease inhibitors. For SDS/PAGE, 50 μg of protein was loaded. Blots were probed with PTEN, phospho-AKT, AKT, phospho-GSK-3 (Cell Signaling Technology, Beverly, MA), and FA synthase (FAS) from BD Bioscience. Same membranes (Bio-Rad) were also probed with actin or vinculin (Sigma) for loading controls.

Northern Blot Analysis. RNA samples were extracted from fresh liver by using TRIzol reagents. For RNA blot, 10 μg of RNA per sample was loaded. Blots were probed with phospho*enol*pyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), and actin for loading control. Probes for PEPCK and G6Pase are generous gifts from E. G. Beale (Texas Tech University, Lubbock, TX) and K. Van Auken (University of Colorado, Denver), respectively.

Statistical Analysis. All data are subjected to statistical analysis by using the EXCEL DATA ANALYSIS TOOL PAK (Microsoft). A Student's *t* test was used to determine the differences between the WT and mutant groups. A *P* value of 0.05 or less was considered significant.

Results

Liver-Specific *Pten* Deletion. We crossed $Pten^{loxP/loxP}$ mice with hepatocyte specific Alb-Cre-transgenic (23) mice to achieve liver-specific *Pten* deletion. The breeding strategy is illustrated in Fig. 1*A*. To avoid potential variations contributed by gender and genetic background, male mice from the F_2 generation, $Pten^{loxp/loxp};Alb\text{-}Cre^{+}$ (mutant) and $Pten^{loxp/loxp};Alb\text{-}Cre^{-}$ (WT), were used for studies described below. PCR analysis of $Pten^{loxp/loxp};Alb\text{-}Cre^{+}$ mice showed that *Pten* deletion, as indicated by excision of the *Pten* locus ($Pten^{\Delta 5}$), is specific to liver with no leakage to other tissues, including white and brown adipose tissues (Fig. 1*B*), which is consistent with the previous report (23). As a result of *Pten* deletion, we observed hyperphosphorylation of AKT in the mutant livers (Fig. 1*C*; P-AKT). A Residual amount of PTEN observed in mutant livers was possibly contributed by cell types other than hepatocytes (Fig. 1*C* and ref. 23).

***Pten* Deletion in the Liver Results in Hepatomegaly, Fatty Liver, and Increased Glycogen Synthesis.** Examination of the mutant liver revealed pale color and marked hepatomegaly (Fig. 2*A Left Upper*). Concomitant with increased liver size, the liver weight and the ratio of liver weight to body weight in the mutant animals were also significantly increased (Fig. 2*A Lower*, $P \leq 0.05$). Histological analysis demonstrated significant morphological changes of mutant livers. The mutant hepatocytes were distended by large cytoplasmic vacuoles that push the nucleus against the cell membrane (Fig. 2*B*, arrows in *Top Right*), although the general lobular architecture remained. Because AKT activation could increase glycogen synthesis as well as FA synthesis, we stained liver sections with periodic acid Schiff's to visualize glycogen, and Oil red O for FAs. Staining with periodic acid Schiff's demonstrated increased glycogen storage in the mutant livers (Fig. 2*B Middle Right*). Strikingly, significant lipid deposition, as indicated by Oil red O staining, was observed in the mutant livers but not in age- and genetic background-matched WT mice (Fig. 2*B Bottom Right*). Quantification of liver TG content revealed a 3-fold increase in the mutant liver (Fig. 2*A Right Lower*), further confirming the fatty liver phenotype.

The progression of fatty liver phenotype appeared to be age-dependent. At 1 month of age, the mutant hepatocytes appeared swollen with minimal lipid deposition, starting from the area surrounding the central terminal hepatic venule (Fig. 1*A Top*). By 3 months of age, a substantial amount of lipid accumulation in the mutant hepatocytes was clearly evident, which further progress to entire liver by 6 months (Fig. 7, which is published as supporting information on the PNAS web site). Infiltration of inflammatory cells and mild fibrosis were observed in some but not all mutant livers at 6 months of age (data not shown). No apparent histological changes were observed in other tissues.

***Pten* Deletion Enhances the FA Synthesis in the Mutant Liver.** To understand the mechanisms underlying liver steatosis, we investigated the sources of lipid in the mutant hepatocytes. Accumulation of fat in the liver could be multifactorial, including increased uptake from peripheral and/or enhanced *de novo* FA

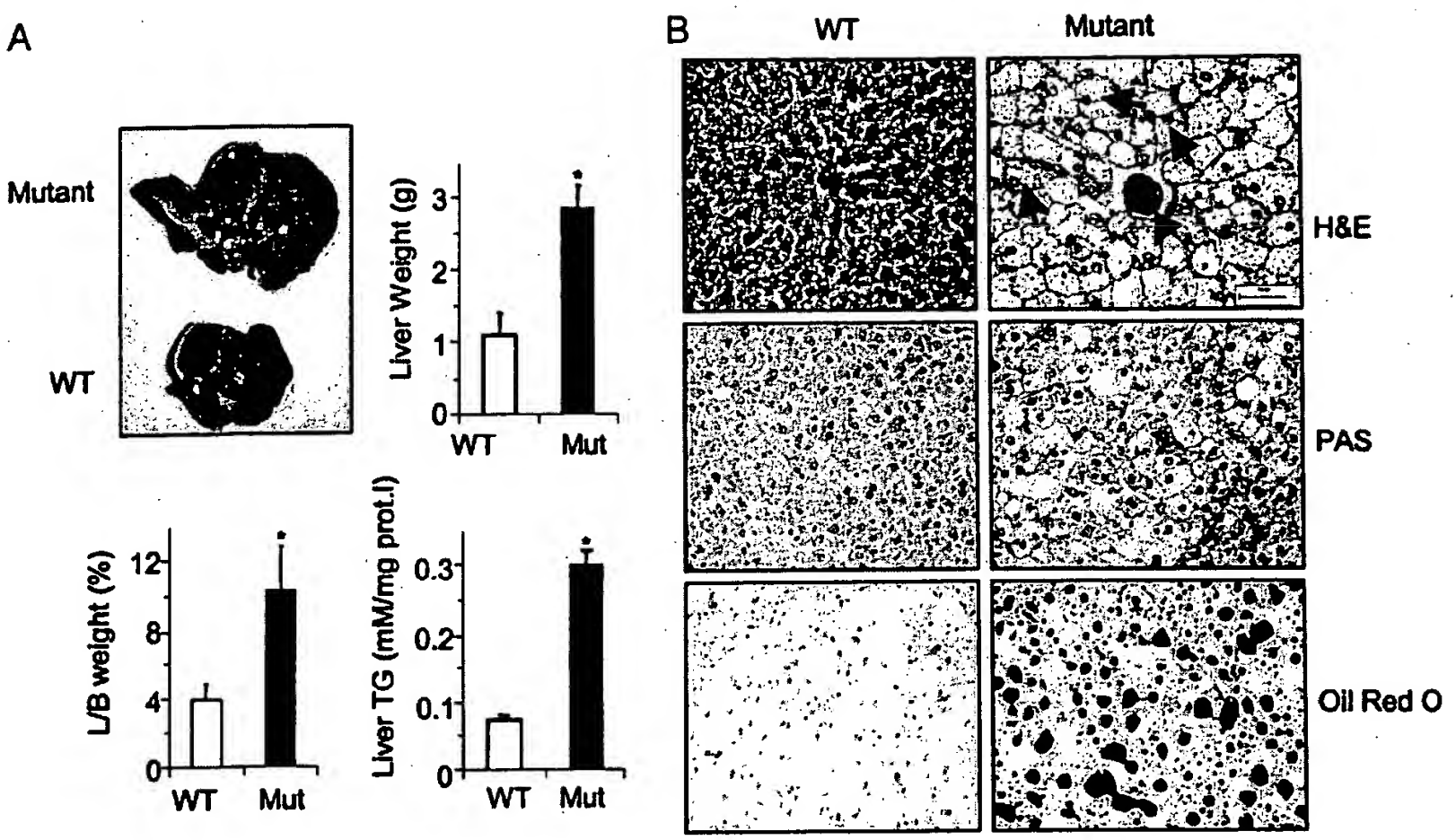

Fig. 2. *Pten* deletion results in hepatomegaly and liver steatosis. (*A*) Hepatomegaly of mutant mice. Photo shows livers from mutant (*Upper*) and WT mouse (*Lower*); mutant mice have heavier livers (*Upper Right*; $n = 6$), increased liver to body weight ratio (*Lower Left*; $n = 6$), and increased TG storage (*Lower Right*; $n = 6$). *, $P \leq 0.05$. (*B*) Histological analysis of liver sections from WT and mutant mice. (*Top*) Hematoxylin/eosin staining. (*Middle*) Periodic acid Schiff's staining (*Bottom*) Oil red O staining. (Bar, 50 μM.)

synthesis by the hepatocytes. We first investigated whether PTEN loss may cause increased FA uptake by isolating primary hepatocytes from 3-month-old WT and mutant mice and examining FA uptake by using fluorescent labeled long-chain FA analogue and fluorescence-activated cell sorter analysis. FAs enter hepatocytes through either passive diffusion (low uptake) or active transportation (high uptake) (28), which can be easily separated by fluorescence-activated cell sorter analysis (24). In WT livers, ≈80% of the hepatocytes are in the low-uptake group. However, the mean uptake of the entire hepatocyte population depends on the high-uptake group because their uptake rates are two orders of magnitude higher than that of the low-uptake group (24). In mutant hepatocytes, uptake by the low-uptake group was significantly increased by 20%, whereas no significant difference was observed in the high-uptake group (Fig. 3*A*). As a net result, total FA uptake by the mutant hepatocytes was not significantly altered. This result was further supported by measuring the levels of two major liver FA transports, FATP2 and FATP5 (28). No significant differences were observed in the protein levels of these two transporters (data not shown).

Increased *de novo* lipogenesis is the other way in which FA can accumulate in the liver. To separate the direct effect of PTEN on FA synthesis from accumulated pathological effects of liver steatosis in aged animals, only 1-month-old male mice were used. We measured the rate of *de novo* FA synthesis by using D_2O and GC/MS (25, 26). Deuterium incorporation into newly synthesized FAs provides a sensitive measurement for the rate of *de novo* lipogenesis. We demonstrated that the rate of FA synthesis is 2.5-fold higher in the mutant livers (Fig. 3*B Left*, $P \leq 0.05$), as compared with the WT livers. To assess whether the newly synthesized FAs could be released to plasma, we measured lipid secretion rate by injecting triton. Lipid output by hepatocytes is mediated by lipoproteins that complex with various lipid molecules to form lipolipoprotein particles. Triton coats these lipid particles (27) and inhibits their peripheral absorption. The plasma TG levels in the mutant mice were higher than that of the WT mice at every time point measured after triton injection (Fig. 3*B Right*; $P \leq 0.05$), suggesting that *Pten* deletion leads to higher rates of both FA synthesis and secretion.

Liver Steatosis Is Accompanied by Decreased Body Fat Content. As a result of *Pten* deletion in the liver, we also observed a 50% reduction of total body fat content (Fig. 3*C Left Upper*) as measured with NMR (29). Consistent with this sensitive *in vivo* measurement, the mutant animals at 1 month of age also showed decreased serum leptin levels (Fig. 3*C Left Lower*). No changes in feeding behavior, as measured by daily food intake, was observed that could account for the declined leptin in mutant mice, because animals from either group consumed 3 ± 0.5 g per animal per day. Interestingly, despite normal levels of plasma TG in the mutant animals (Fig. 3*C Right Lower*), there is a 30% decrease in circulating free FAs (NEFA) levels (Fig. 3*C Right Upper*), which is most likely due to decreased lipolysis rate, as measured by the *in vivo* lipolysis assay (Fig. 8, which is published as supporting information on the PNAS web site). Taken together, these results suggest that *Pten* liver-specific deletion may lead to redistribution of fat from other tissues to the liver.

Liver-Specific Deletion of *Pten* Causes Decreased Fasting Glucose Levels and Improved Glucose Tolerance. Because the levels of NEFA are thought to have a significant influence on insulin sensitivity, we measured both basal glucose levels and the rates of glucose clearance on animals at 1, 3, and 6 months of age. Deleting *Pten* caused a decrement in fasting plasma glucose levels in 1- and 3-month-old mice (Fig. 4*A Left* and *Center*, $P \leq 0.05$). This occurrence of lowered glucose concentration was accompanied by a significant decrease in fasting plasma insulin levels (Fig. 4*B Left*). At 6 months of age, when severe steatosis was observed in the liver, the fasting glucose level in the mutant

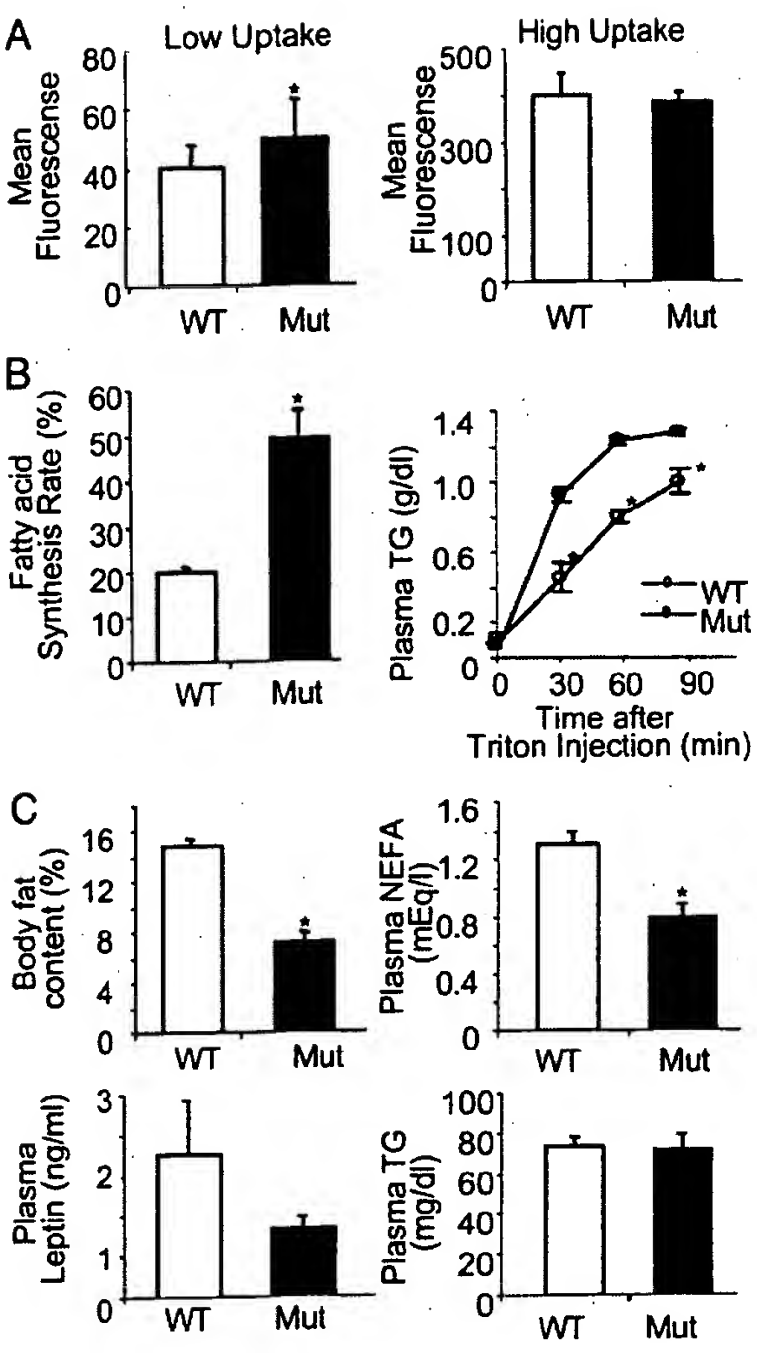

Fig. 3. *Pten* deletion enhances FA synthesis and secretion by hepatocytes. (*A*) Uptake of long-chain FA by WT and mutant primary hepatocytes. (*Left*) Low-uptake group. (*Right*) High-uptake group. $n = 3$. (*B*) FA synthesis rate is calculated as newly synthesized portion of palmitate (*Left*, $n = 6$). Lipid secretion rate is measured as plasma TG levels at indicated time points after triton injection (*Right*, $n = 3$). (*C*) Lipid indexes of 1-month-old *Pten* WT and mutant mice: (*Left Upper*) body fat content of WT ($n = 7$) and mutant mice ($n = 6$). (*Right Upper*) Plasma NEFA levels in WT ($n = 6$) and mutant ($n = 6$) mice. (*Left Lower*) Plasma leptin levels in WT ($n = 5$) and mutant mice ($n = 5$). (*Right Lower*) Plasma TG levels in WT ($n = 5$) and mutant mice ($n = 5$). *, $P \leq 0.05$.

mice was comparable with that of the WT mice (Fig. 4*A Right*), suggesting that even though the insulin level in the mutant remained lower than WT mice (Fig. 4*B Right*), accumulated liver damage has started to influence liver function.

When challenged with an i.p. glucose load, the mutant mice demonstrated a lower peak glucose concentration at 15 min (by 30%, $P \leq 0.05$) and a faster decline of plasma glucose levels throughout the glucose tolerance curve (Fig. 5*A*, $P \leq 0.05$). The increased glucose clearance during an i.p. glucose load occurred in all age groups in mutant mice (Fig. 5) but is most evident in mice at 1 month of age: plasma glucose levels in mutants returned to baseline between 60 and 120 min, whereas it remained above baseline at 120 min in WT animals. These results suggest that *Pten* deletion in the liver not only causes increased insulin action in the liver, as indicated by increased glycogen and FA deposition, but also enhances glucose disposal.

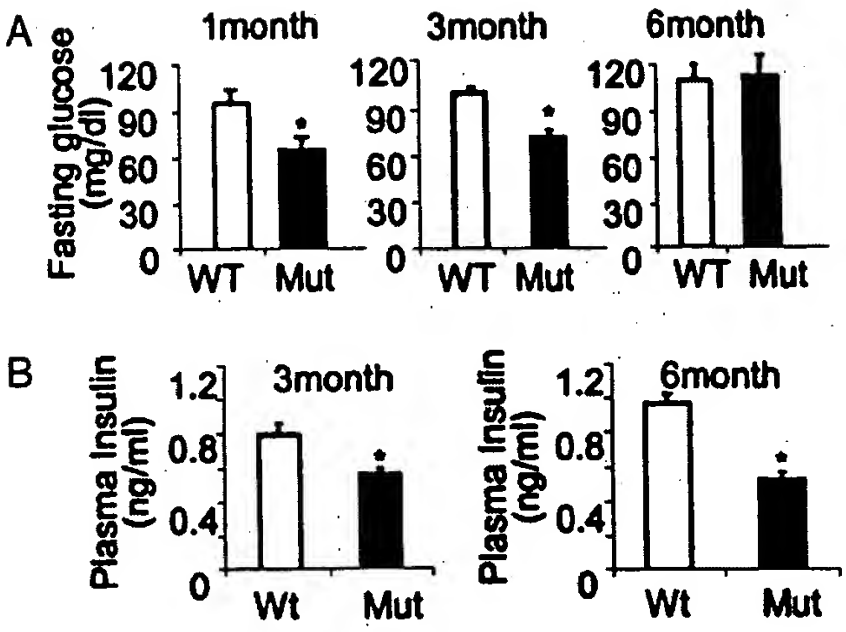

Fig. 4. Fasting plasma glucose and insulin levels. (*A*) Fasting plasma glucose levels in 1- (*Left*), 3- (*Center*), and 6-month-old (*Right*) mice. Open bars, WT ($n = 9$); filled bars, mutant ($n = 11$). (*B*) Fasting plasma insulin concentrations in 3- (*Left*) and 6-month-old (*Right*) mice. $n = 6$. *, $P \leq 0.05$.

***Pten* Regulates Key Enzymes Controlling Glycogen and FA Syntheses.** To correlate between the metabolic phenotypes and alterations of insulin-controlled signaling pathway in mutant liver, we investigated the expression of a number of genes involved in glucose and lipid metabolism. GSK-3 is the key molecule negatively regulating glycogen synthase (GS), which is required for the incorporation of glucose into glycogen. GSK-3 constitutively phosphorylates GS to inactive its enzymatic activity. Phosphorylation of GSK-3 by AKT inactivates the kinase and relieves its block on GS (30). We showed that phosphorylation of GSK-3β was moderately increased (50%) when *Pten* is deleted (Fig. 6*A*). Thus, GSK-3β hyperphosphorylation may account for the increased glycogen accumulation observed in mutant livers. Deletion of *Pten* resulted in a marked increase in liver FAS levels (Fig. 6*A*), which is likely to play an important role in the increased *de novo* FA synthesis observed in the mutant livers. In contrast, there was down-regulation of two key gluconeogenic enzymes in the mutant mice, namely glucose-6-phosphatase (G6Pase) and PEPCK. The expression of these two enzymes was similarly decreased in the mutant livers although the change in PEPCK was more pronounced (Fig. 6*B*). Together, the changes in FAS, G6Pase, and PEPCK enzyme levels may divert substrate toward FA synthesis.

Discussion

Because T2D is characterized by an impaired insulin action, research efforts have focused on understanding the insulin-

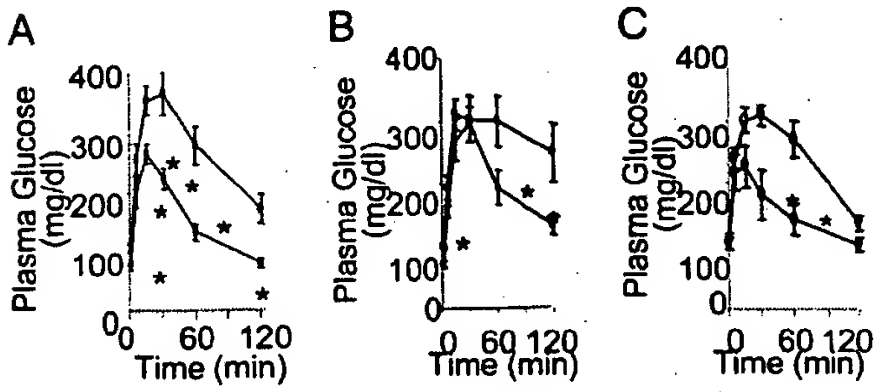

Fig. 5. i.p. GTT. (*A*) One-month GTT with WT ($n = 9$) and mutant ($n = 11$) mice. (*B*) Three-month GTT with WT ($n = 6$) and mutant ($n = 8$) mice. (*C*) Six-month GTT with WT ($n = 11$) and mutant ($n = 8$) mice. *, $P \leq 0.05$

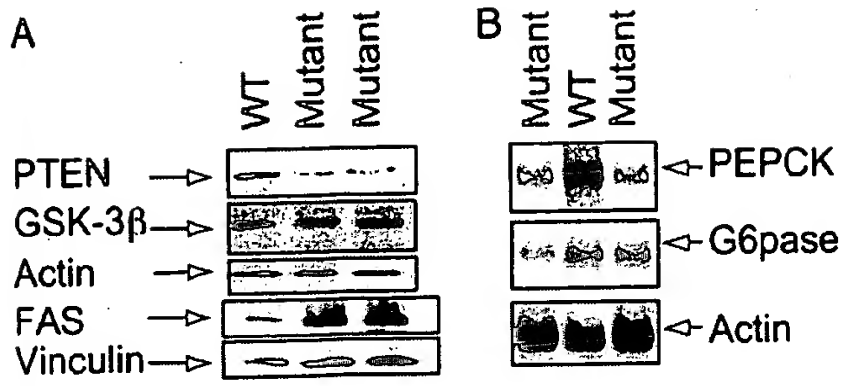

Fig. 6. *Pten* deletion results in inhibition of gluconeogenesis and activation of lipogenesis enzymes in the liver. (*A*) Western blot analysis of GSK-3β and FAS. Blots were probed with PTEN (first section), phospho-GSK-3β (second section), and FAS (fourth section). Actin and vinculin were used as loading controls. (*B*) Northern blot analysis of PEPCK and G6Pase gene expressions in mouse liver. Actin was used as loading control.

signaling pathway in an attempt to identify suitable therapeutic target(s) for drug intervention. Although significant progress has been made in the understanding of insulin activation, less is known about the negative control mechanisms that result in returning the activated IR to basal level. Because IR has intrinsic tyrosine kinase activity, various protein tyrosine phosphatases (PTPs) have been studied and implicated in controlling insulin receptor dephosphorylation and inactivation (31). In this study, we provided evidence that PTEN, a lipid phosphatase, serves as an important negative modulator for the insulin-signaling pathway by antagonizing the PI3-kinase/AKT signaling pathway. Thus, the role of PTEN in regulating insulin signaling pathway is evolutionary conserved from *Caenorhabditis elegans* (12) to mammals.

Deletion of *Pten* in the liver led to increased insulin sensitivity in the liver and improved overall glucose tolerance. Not surprisingly, the *Pten* liver deletion mouse shares similarity to PTP-1B knockout mouse. PTP-1B is a tyrosine phosphatase that negatively regulates IR signaling by presumably dephosphorylating IR, IRSs, or possibly other phosphotyrosyl molecules in the insulin-signaling pathway. PTP-1B deficiency causes insulin sensitivity, as demonstrated by lower glucose levels and improved glucose clearance in an i.p. glucose load (32), similar to what we observed in the liver-specific *Pten* deletion mouse. In contrast, phenotype associated with *Pten* liver-specific deletion is completely opposite to the insulin-resistant phenotype observed in the liver IR knockout mouse, which consists of severe liver insulin resistance and mild diabetes with hyperinsulinemia (3). Thus, our study provide, to our knowledge, the first genetic evidence for the role of PTEN in controlling the insulin-signal pathway in mammals *in vivo* and suggests that perturbing the functions of the negative regulators of the insulin-signaling pathway, either at the receptor level, as PTP-1B for IR, or on its downstream mediators, as PTEN for PI3-kinase/AKT, may lead to increased insulin sensitivity.

Lipid metabolism is also affected in *Pten* mutant mice. Similar to PTP-1B mice, which have increased energy expenditure and decreased adiposity (33), the *Pten* liver-specific deletion mice have decreased total body fat, and serum NEFA and leptin levels. Whether PTEN liver deficiency will lead to increase energy expenditure and resistance to diet-induced obesity, as reported for PTP-1B$^{-/-}$ mice, remains to be investigated. Furthermore, it is not clear whether lean body mass and hepatosteatosis in *Pten* mutant mice may alter the production and signaling of adiponectin, an adipocyte hormone involved in whole-body energy expenditure (34–36).

Unlike the PTP-1 knockout mouse, deleting *Pten* in the liver led to the development of hepatomegaly and fatty liver. Fatty liver has also been observed in *ob/ob* mice, a type II diabetes model (37, 38). Several fatty liver (nonalcoholic fatty liver disease) models exist, including the *fa/fa* rats and the lipoatrophic mice (39–41). The common denominator for these fatty liver models is hyperinsulinemic and insulin resistance, despite the differences in plasma leptin level and adipose tissue mass. In humans, liver steatosis is known to associate with obesity and diabetes conditions with hyperinsulinemia and insulin resistance (42). It was postulated that the high level of NEFA in circulation due to peripheral insulin resistance is the primary cause of fatty liver (43, 44). We showed in *Pten* mutants, a different fatty liver phenotype from that of hyperinsulinemic and insulin-resistant models. Fatty liver in our *Pten* mutant is associated with relative hypoinsulinemia, enhanced insulin action and low plasma NEFA, which is concomitant with lipoatrophy. Thus, our model has provided an insight into the mechanisms responsible for liver steatosis.

Recent studies (45–47) also suggest that the interaction among the different insulin-sensitive organs may be mediated through interactions between insulin and leptin signaling pathways, which may converge onto AKT and its effectors, such as the forkhead transcription factors and other not-yet-identified targets (48). In the *ob/ob* or knockout models, insulin resistance accompanies defects in leptin action in the liver (2–4, 38, 41, 47). *In vivo*, PTP-1B regulates leptin signal transduction, likely by targeting Jak2 (49). Interestingly, in the *Pten* liver knockout, increased insulin sensitivity is accompanied by hypoleptinemia.

The interplay between glucose and lipid metabolism is mediated by the reciprocal relationship of substrate use and distribution between liver and peripheral tissues. This relationship was observed in the IR/glucose transporter knockout mice with decreased glucose utilization (2, 4, 50), or in the *ob/ob* model with increased lipogenesis (38). In our study, the hepatosteatosis phenotype was accompanied by improved glucose tolerance and leaner body mass in mutant mice. In this case, enhanced insulin signaling in the liver resulted in redistribution of body fat from fatty tissue to the liver. The liver also appeared to be using more glucose for lipogenesis, as evidenced by the reduced levels of gluconeogenic enzymes. As a result, liver consumes more energy to synthesize and store fat. Thus, liver became the sacrificial organ to maintain glucose control and lean body mass.

In summary, we have created a valuable model for studying insulin action in the liver, as well as the complex interaction among insulin-sensitive organs and the reciprocal regulations of glucose and lipid metabolisms. The similarities shared by *Pten*-deficient mice and PTP-1B$^{-/-}$ mice further emphasize the importance of the negative regulators in normal insulin signaling and in the development of T2D and suggest that PTEN may be an attractive candidate or target for inhibitors as therapeutic intervention in the treatment of T2D.

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1. Baudry, A., Leroux, L., Jackerott, M. & Joshi, R. L. (2002) *EMBO Rep.* 3, 323–328.
2. Bruning, J. C., Michael, M. D., Winnay, J. N., Hayashi, T., Horsch, D., Accili, D., Goodyear, L. J. & Kahn, C. R. (1998) *Mol. Cell* 2, 559–569.
3. Michael, M. D., Kulkarni, R. N., Postic, C., Previs, S. F., Shulman, G. I., Magnuson, M. A. & Kahn, C. R. (2000) *Mol. Cell* 6, 87–97.
4. Abel, E. D., Peroni, O., Kim, J. K., Kim, Y. B., Boss, O., Hadro, E., Minnemann, T., Shulman, G. I. & Kahn, B. B. (2001) *Nature* 409, 729–733.
5. Zisman, A., Peroni, O. D., Abel, E. D., Michael, M. D., Mauvais-Jarvis, F., Lowell, B. B., Wojtaszewski, J. F., Hirshman, M. F., Virkamaki, A., Goodyear, L. J., *et al.* (2000) *Nat. Med.* 6, 924–928.

6. Koteish, A. & Mae Diehl, A. (2002) *Best Pract. Res. Clin. Gastroenterol.* **16**, 679–690.
7. Accili, D., Kido, Y., Nakae, J., Lauro, D. & Park, B. C. (2001) *Curr. Mol. Med.* **1**, 9–23.
8. Huisamen, B., van Zyl, M., Keyser, A. & Lochner, A. (2001) *Mol. Cell. Biochem.* **223**, 15–25.
9. Nakae, J., Kitamura, T., Silver, D. L. & Accili, D. (2001) *J. Clin. Invest.* **108**, 1359–1367.
10. Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., Crenshaw, E. B., III, Kaestner, K. H., Bartolomei, M. S., Shulman, G. I. & Birnbaum, M. J. (2001) *Science* **292**, 1728–1731.
11. Sun, H., Lesche, R., Li, D. M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X. & Wu, H. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 6199–6204.
12. Ogg, S. & Ruvkun, G. (1998) *Mol. Cell* **2**, 887–893.
13. Mihaylova, V. T., Borland, C. Z., Manjarrez, L., Stern, M. J. & Sun, H. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 7427–7432.
14. Rouault, J. P., Kuwabara, P. E., Sinilnikova, O. M., Duret, L., Thierry-Mieg, D. & Billaud, M. (1999) *Curr. Biol.* **9**, 329–332.
15. Gil, E. B., Malone Link, E., Liu, L. X., Johnson, C. D. & Lees, J. A. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 2925–2930.
16. Stiles, B., Gilman, V., Khanzenzon, N., Lesche, R., Li, A., Qiao, R., Liu, X. & Wu, H. (2002) *Mol. Cell. Biol.* **22**, 3842–3851.
17. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P. & Mak, T. W. (1998) *Cell* **95**, 29–39.
18. Di Cristofano, A., Pesce, B., Cordon-Cardo, C. & Pandolfi, P. P. (1998) *Nat. Genet.* **19**, 348–355.
19. Suzuki, A., de la Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., *et al.* (1998) *Curr. Biol.* **8**, 1169–1178.
20. Podsypanina, K., Ellenson, L. H., Nemes, A., Gu, J., Tamura, M., Yamada, K. M., Cordon-Cardo, C., Catoretti, G., Fisher, P. E. & Parsons, R. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 1563–1568.
21. Butler, M., McKay, R. A., Popoff, I. J., Gaarde, W. A., Witchell, D., Murray, S. F., Dean, N. M., Bhanot, S. & Monia, B. P. (2002) *Diabetes* **51**, 1028–1034.
22. Lesche, R., Groszer, M., Gao, J., Wang, Y., Messing, A., Sun, H., Liu, X. & Wu, H. (2002) *Genesis* **32**, 148–149.
23. Postic, C. & Magnuson, M. A. (2000) *Genesis* **26**, 149–150.
24. Stahl, A., Hirsch, D. J., Gimeno, R. E., Punreddy, S., Ge, P., Watson, N., Patel, S., Kotler, M., Raimondi, A., Tartaglia, L. A. & Lodish, H. F. (1999) *Mol. Cell* **4**, 299–308.
25. Lee, W. N., Bassilian, S., Guo, Z., Schoeller, D., Edmond, J., Bergner, E. A. & Byerley, L. O. (1994) *Am. J. Physiol.* **266**, E372–E383.
26. Lee, W. N., Bassilian, S., Lim, S. & Boros, L. G. (2000) *Am. J. Physiol.* **279**, E425–E432.
27. Li, X., Catalina, F., Grundy, S. M. & Patel, S. (1996) *J. Lipid Res.* **37**, 210–220.
28. Stahl, A., Gimeno, R. E., Tartaglia, L. A. & Lodish, H. F. (2001) *Trends Endocrinol. Metab.* **12**, 266–273.
29. Barac-Nieto, M. & Gupta, R. K. (1996) *J. Magn. Reson. Imaging* **6**, 235–238.
30. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. & Hemmings, B. A. (1995) *Nature* **378**, 785–789.
31. Asante-Appiah, E. & Kennedy, B. P. (2003) *Am. J. Physiol.* **284**, E663–E670.
32. Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A. L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C. C., *et al.* (1999) *Science* **283**, 1544–1548.
33. Klaman, L. D., Boss, O., Peroni, O. D., Kim, J. K., Martino, J. L., Zabolotny, J. M., Moghal, N., Lubkin, M., Kim, Y. B., Sharpe, A. H., *et al.* (2000) *Mol. Cell. Biol.* **20**, 5479–5489.
34. Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., *et al.* (1999) *Biochem. Biophys. Res. Commun.* **257**, 79–83.
35. Matsubara, M., Katayose, S. & Maruoka, S. (2003) *Eur. J. Endocrinol.* **148**, 343–350.
36. Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., *et al.* (2003) *Nature* **423**, 762–769.
37. Austin, B. P., Garthwaite, T. L., Hagen, T. C., Stevens, J. O. & Menahan, L. A. (1984) *Exp. Gerontol.* **19**, 121–132.
38. Yang, S. Q., Lin, H. Z., Mandal, A. K., Huang, J. & Diehl, A. M. (2001) *Hepatology* **34**, 694–706.
39. Shimomura, I., Hammer, R. E., Richardson, J. A., Ikemoto, S., Bashmakov, Y., Goldstein, J. L. & Brown, M. S. (1998) *Genes Dev.* **12**, 3182–3194.
40. Bluher, M., Michael, M. D., Peroni, O. D., Ueki, K., Carter, N., Kahn, B. B. & Kahn, C. R. (2002) *Dev. Cell* **3**, 25–38.
41. Chanussot, F., Ulmer, M., Ratanasavanh, R., Max, J. P. & Debry, G. (1984) *Int. J. Obes.* **8**, 259–270.
42. Clark, J. M. & Diehl, A. M. (2002) *Curr. Diab. Rep.* **2**, 210–215.
43. Petersen, K. F., Oral, E. A., Dufour, S., Befroy, D., Ariyan, C., Yu, C., Cline, G. W., DePaoli, A. M., Taylor, S. I., Gorden, P. & Shulman, G. I. (2002) *J. Clin. Invest.* **109**, 1345–1350.
44. Kraegen, E. W., Clark, P. W., Jenkins, A. B., Daley, E. A., Chisholm, D. J. & Storlien, L. H. (1991) *Diabetes* **40**, 1397–1403.
45. Goetze, S., Bungenstock, A., Czupalla, C., Eilers, F., Stawowy, P., Kintscher, U., Spencer-Hansch, C., Graf, K., Nurnberg, B., Law, R. E., *et al.* (2002) *Hypertension* **40**, 748–754.
46. Carvalheira, J. B., Ribeiro, E. B., Folli, F., Velloso, L. A. & Saad, M. J. (2003) *Biol. Chem. Hoppe-Seyler* **384**, 151–159.
47. Bruning, J. C., Gautam, D., Burks, D. J., Gillette, J., Schubert, M., Orban, P. C., Klein, R., Krone, W., Muller-Wieland, D. & Kahn, C. R. (2000) *Science* **289**, 2122–2125.
48. Guo, S., Rena, G., Cichy, S., He, X., Cohen, P. & Unterman, T. (1999) *J. Biol. Chem.* **274**, 17184–17192.
49. Zabolotny, J. M., Bence-Hanulec, K. K., Stricker-Krongrad, A., Haj, F., Wang, Y., Minokoshi, Y., Kim, Y. B., Elmquist, J. K., Tartaglia, L. A., Kahn, B. B. & Neel, B. G. (2002) *Dev. Cell* **2**, 489–495.
50. Kim, J. K., Zisman, A., Fillmore, J. J., Peroni, O. D., Kotani, K., Perret, P., Zong, H., Dong, J., Kahn, C. R., Kahn, B. B. & Shulman, G. I. (2001) *J. Clin. Invest.* **108**, 153–160.